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THE DISTRIBUTION OF IRON IN BLOOD.

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MUCH of the confusion regarding the distribution of iron in blood is attributable to the lack of a reliable method of quantitative analysis. Various colorimetric methods have been described in recent years, and experience has shown that most of them are, if anything, less accurate than the older volumetric methods which they were meant to supersede.

The estimation of the total iron in whole blood has been used as a means of determining the hæmoglobin concentration. The existence of iron in the plasma and within the erythrocytes has been conceded, but dismissed as negligible in amount; upon what grounds is not stated. In America Haden (1933) has used Kennedy's method (1927) for calibrating his hæmoglobinometer, finding close agreement with gas analysis. Murphy, Lynch and Howard (1931) also employed the method in conjunction with the Newcomer disc for determining the iron volume index. These latter workers state that they have reason to believe that the method for estimating the iron gives too low a reading. The whole blood iron content and the Hb concentration of 18 normal males is given, and on calculating the Fe present in the recorded Hb readings one finds that it is consistently about 8 p.c. more than the total iron determined. The method of calculation will be discussed later.

Alt (1934) tested the accuracy of various hæmoglobinometers by means of Wong's new method (1928), basing his work on the common assumption. He found that the average error for Sahli was 2·7 p.c., Newcomer 3·4 p.c., and Dare 3·9 p.c. Reich and Tiedeman (1932), who investigated the iron volume index by means of a thiocyanate colorimetric method, record that in some cases the Hb exceeded the equivalent iron. Helmer and Emerson (1934) found that Kennedy's method gave results in close agreement with those obtained by gas analysis and concluded that whole blood Fe could be used

for Hb determinations. Sachs, Levine and Appelsis (1933) reached a similar conclusion. On the other hand Sobel and Drekter (1933) with a thiocyanate method based on that of Wong obtained results which forced them to conclude that there was no constant relation between Hb and Fe concentrations ; in 88 p.c. of their 135 cases the Hb by Newcomer's method exceeded that calculated from the Fe content of whole blood.

In contrast to all the foregoing stands the work of Barkan (1927, 1933), who found that the whole blood iron was distributed between the plasma (0·5 p.c.) and the erythrocytes. The latter contained more iron than was held as Hb. He found further that this loosely bound iron is not adsorbed by aluminium hydrate as is that of the plasma and Hb. Barkan's observations have been substantially confirmed by Winegarden and Borsook (1933), who used a method of dialysis through cellophane, and found that the loosely bound non-Hb Fe was 4 to 6 p.c. of the whole blood Fe.

We have examined Kennedy's method, which is no worse than a number of similar methods, and find it to be inaccurate. In a search for a reliable and rapid method attention was first directed to the method of ashing. Simple dry ashing results in the loss of ferric chloride by volatilization, which begins at 110° C. Farrar (1935) prevents volatilization by dry ashing in the presence of calcium carbonate in a platinum or quartz crucible. The pyrophosphates formed are hydrolyzed with weak HCl for 24 hours. We found that there was some interaction between the quartz and the potassium present in the blood, and in any event the method was tedious owing to the time required for hydrolysis.

Wet ashing seemed preferable, and we found that an air-cooled condenser prevented the loss of ferric chloride and also conserved the acid, the importance of which will be apparent later. Wet ashing in an open flask is widely used, and we found that the loss of iron noted by Farrar could be demonstrated with ease by holding over the mouth of the flask a filter-paper moistened with sodium thiocyanate solution ; the paper turns pink at once. The risk of loss must be increased with Kennedy's method because of the use of perchloric acid with its end-product hydrochloric acid. When the condenser was in use no iron could be detected at its mouth.

The ashed solution having been obtained, various methods of determining the Fe present were considered. The volumetric methods were found too tedious for routine use if a large number of specimens were to be examined. Neumann's method (1902) requires precipitation of the Fe in the ashed solution with an alkali ; as few salts are completely insoluble tests were made with small quantities of Fe, and it was found that the Fe was not completely precipitated. Most of these methods require preliminary reduction of the ferric salt to the ferrous state, and we found that the use of metals such as zinc or magnesium involved the formation of much additional salt, which interfered with permanganate titration and with a modified spotting method using potassium ferricyanide. This objection was overcome by the use of palladium. But if palladium is used in the ordinary manner the nascent hydrogen available is too small for the total reduction of the ferric iron. We therefore devised an apparatus which we describe as the hollow palladium cathode. In this a large piece of Pd foil is shaped to the form of a shallow cup with a flat bottom.

The ferric salt is placed in it and the cup immersed to 2 or 3 mm. depth in an acid electrolytic bath. The cup is connected to the negative pole of a battery. and the positive pole to a large piece of platinum foil which is the anode. A current of 6 to 8 amp. at a pressure of 5 or 6 v. is passed for about 75 min. and results in the complete reduction of the ferric salts. The advantage of this apparatus is that the supply of nascent hydrogen is unlimited and it is the only method of reduction which does not introduce additional salt. Nevertheless we abandoned it because of the unsatisfactory end-point in subsequent titration, and turned to colorimetric methods in the hope of more rapid determination. The hollow palladium cathode has been described as it is thought it may be found useful in other biological fields.

In colorimetry the thiocyanate reaction is commonly used. It is a highly sensitive qualitative test for ferric iron ; it is also the classic example of the reversibility of chemical action. Amyl alcohol is advocated as a means of minimizing the reverse action, but from a series of experiments we are satisfied that it is of little use. The colour originally developed is influenced to a large extent by the relative concentrations of Fe and thiocyanate salt, consequently small Fe concentrations read too high and strong concentrations too low. The colour is also influenced by the concentration of other salts present, and since Hb is the principal protein of blood, the ammonium sulphate formed must vary with the Hb concentration. The most powerful decolorizing factor is sulphuric acid ; nitric acid has been advocated as a means of preventing loss of colour, but it is not very effective. Amyl alcohol does nothing to prevent acid decolorization. Owing to volatilization the concentration of sulphuric acid in Kennedy's method is unknown and uncontrollable without the aid of a condenser. We therefore conclude that the thiocyanate reaction is unsuitable for colorimetry.

Other agents which developed colour with iron were investigated. Di-*iso*nitrosoacetone (Dubsky and Kuras, 1929) requires ferrous reduction, and takes two hours to develop its full colour. Potassium molybdenocyanide, said to develop an intense blue with ferric salts, was unobtainable. Pulsifer (1904) describes a colorimetric reaction with acetyl acetone for ferric salts, but we found the colour not sufficient for the concentration found in biological material. Protocatechuic acid develops a deep colour, but the hue is too dependent upon the concentration of alkali. Yeo (1932) advocates 7-iodo-8-hydroxyquinoline-5-sulphonic acid, but we found it required too delicate adjustment of the pH to be satisfactory. Reis and Chakmajian (1931) used the prussian blue reaction with gum acacia to hold the insoluble salt in suspension ; in view of the light dispersed by suspended particles it does not suggest that a linear function would be obtained. We tried the soluble prussian blue reaction, but found it took too long to develop its full colour and was liable to precipitation by salts ; it also required preliminary ferrous reduction.

Thioglycollic acid appeared to offer most promise. It was first described by Andreasch (1879), and for colorimetry by Lyons (1927). Leavell and Ellis (1934) tested it against the thiocyanate reaction and found that it gave 2·7 p.c. higher mean reading. The thioglycollic reaction has also been examined by Burmester (1934), who found that for colorimetry it was as accurate as titanium titration, and more accurate than thiocyanate. Our first experiments with it

soon showed that it was influenced by the concentration of salt solution. This must necessarily be high because the reaction is developed by excess of ammonia, which means that the whole of the acid present must be neutralized. The inhibitory effect of the salt on the intensity of colour is peculiar in that it disappears suddenly and completely when the salt concentration is below a certain value, which appeared to be about 7 p.c. A pale purple colour is developed in high salt concentration which deepens on the addition of water. The increase of colour is abrupt, and at any concentration below this crucial point the colour developed is strictly proportional to the Fe present.

The colour takes a little time to stabilize itself, and the time required varies with the amount of ammonia used, but the final colour is not affected. Moreover, shaking the solution causes a temporary increase of colour, which disappears in a few minutes, leaving the solution at its former intensity. The shaking can be repeated indefinitely and the colour return is always the same. Sodium can be used for partial neutralization, and does not appear to have as great a disturbing influence as ammonia.

In the method about to be described attention has been paid to various minutiæ of technique, and if we seem to labour them it is because experience has shown that they are necessary for the maintenance of a high standard of accuracy. It is frequently advised that the standard be prepared from pure iron wire—a commodity which does not exist. Ferrous ammonium sulphate has long been a standard analytical salt, and there is no reason why it should not be used for biochemical work. We found that a standard prepared from iron wire was 1 to 3 p.c. weaker than that prepared from the double salt.

METHOD.

A. *For Whole Blood.*

The blood is ashed in a round-bottomed 250 c.c. Pyrex flask fitted with a ground-in air-cooled condenser tube, 1 metre long. The efficiency of the tube is increased by heating it at a point about 40 cm. from the lower end and giving it at this point a slight spiral twist, then gently pressing the two ends together in a telescoping fashion ; this produces a constriction in the tube. The portion above the constriction is filled with pieces of glass tubing about 15 mm. long, and of a diameter just large enough to prevent them passing the constriction and falling into the flask.

The air-cooled condenser is very efficient, and allows only water vapour and a small quantity of HCl gas to escape, consequently the ashing can be carried out on the open bench. The acid left in the flask at the end of the ashing is constantly equal to 2·7 c.c. of concentrated sulphuric acid. A water-cooled condenser is no more efficient, and an accident to it might result in a mutilating explosion.

The flask and condenser are mounted upright in a retort stand, one clamp holding the neck of the flask and another the middle of the condenser ; both should be holding loosely to allow for expansion of the apparatus on heating. From a pipette calibrated for the delivery of oxalated blood (Jenkins and Don, 1933) 1·0 c.c. of whole blood is delivered into the flask ; 2·5 c.c. of concentrated

sulphuric acid and 1·0 c.c. of 60 p.c. perchloric acid are added and the flask is then heated over a small flame. The flame should be from a Meker or Bunsen burner, and turned down so that its tip just touches the bottom of the flask. Too rapid heating results in some of the material being thrown up into the condenser, where it may remain until washed out in the later manipulations, with the effect that a turbid solution is produced which gives too high a reading. For similar reasons it is advisable to use a 250 c.c. flask rather than one of 100 c.c. The larger flask has a greater area for radiation, and the mouth of the condenser is further away from the surface of the fluid. When the fluid has become a transparent red or yellow it is permissible to increase slightly the size of the flame.

The ashing should continue until the liquid is either a very pale yellow whilst boiling, which takes about 40 minutes, or it may be allowed to pass beyond that stage until it turns suddenly to a bright greenish yellow ; there is no advantage in carrying it to the second yellow stage. The solution should be quite colourless when cool, and if it retains a yellow tint ashing is incomplete.

When the flask is cold the condenser is detached and washed out with 20 c.c. of distilled water and the washing poured into the flask. The flask is agitated for a moment and the solution poured into a 100 c.c. graduated cylinder. The flask is washed out with another 20 c.c. of water and the latter added to that in the cylinder.

Next add 9·0 c.c. of 40 p.c. NaOH solution, agitate gently to mix and place the cylinder in a large vessel of water to cool. Then add 0·3 c.c. of 90 p.c. thioglycollic acid, mix thoroughly and add 0·880 ammonium hydroxide solution, a drop at a time, until on agitating a faint purple colour is suffused throughout the solution. Add 1·0 c.c. more ammonia and fill up with water to 80 c.c., inverting the cylinder 3 or 4 times to ensure thorough mixing. The solution is then ready for the colorimeter.

Preparation of the standard.—Dissolve 3·529 g. of ferrous ammonium sulphate, of analytical standard of purity, in 500 c.c. of distilled water. Add 2·0 c.c. of concentrated sulphuric acid ; make up to 1 litre. This solution contains 50 mg. of Fe per 100 c.c. From a microburette place 1·0 c.c. of the stock solution in a graduated 100 c.c. cylinder, add 40 c.c. of water, then 2·7 c.c. of concentrated sulphuric acid, mix and cool. Next add 9·0 c.c. of 40 p.c. NaOH solution and continue as for the sample described above.

Reading the colorimeter.—Fill both cups of the colorimeter with the standard, set the plungers at 20 mm., wait 5 minutes, then balance the instrument optically. Next remove the right-hand cup and fill it with the sample, wait 5 minutes, then take the reading. The calculation is obvious. The movement of the plunger does not affect the intensity of colour, and the cup containing the standard can be left untouched, as it will remain constant for many hours.

B. *For Plasma.*

The plasma should be separated by high-speed centrifugalization in order to show the packed cell volume. An arbitrary deduction of 10 p.c. is made from it to obtain an approximation to the true cell volume, as shown by C. E. Jenkins (1932). It is too laborious to undertake an actual determination of

the true volume in addition to the rest of the work, and the error introduced by the empirical allowance is very small even in anæmia.

The estimation of plasma is described because its ascertainment is imperative in the investigation of various types of anæmia, but for normal males it was soon apparent that within very narrow limits the plasma-content of whole blood is 0·3 mg., which agrees with the findings of Marlow and Taylor (1934), who found the amount to range from 0·4 to 0·7 mg. per 100 c.c. of plasma. When normal male blood is being investigated it is therefore justifiable to make an empirical allowance of 0·3 mg. for the plasma Fe. Most of our normal males have had this arbitrary subtraction made; but for pathological cases and normal females an estimation must be made.

Ash a known volume of plasma, 2 to 3 c.c., with 2·5 c.c. of sulphuric acid and 1·0 c.c. of perchloric acid, then proceed as for whole blood. The concentrations are usually so weak that they cannot easily be compared in the colorimeter and another method is used. From the 50-mg. standard take into separate flasks 8, 6, 4 and 2 c.c. To the flasks in the order given add 72, 74, 76 and 78 c.c. of distilled water. Then add one drop each of strong ammonia and thioglycollic acid to each flask. The result is 80 c.c. each of four standards, representing 5, 3·75, 2·5 and 1·25 mg. respectively. The sample is left in its measuring cylinder and a standard is poured into another 100 c.c. cylinder. Since each is 80 c.c. and the length of the column is about equal in each, the colour intensity can be gauged by looking down into the cylinders.

In certain phases of anæmia the Fe concentration is high enough to admit of examination in a colorimeter, in which case some of the 50 mg. standard is diluted to a strength of 10 or 15 mg. The calculation is again obvious, but it is necessary to remember to make due allowance for the volume of plasma ashed. The result can be expressed forthwith as the concentration of Fe in 100 c.c. of plasma, or the concentration in whole blood can be ascertained by taking into account the percentage cell volume.

TESTS OF METHOD.

The method was tested for proportional and absolute accuracy. Two volumes of blood were ashed and compared with one volume of the same blood. They are single determinations, and if duplicates had been done the results would probably have been better.

TABLE I.

Two volumes.	One volume.	Percentage error.
47·0	24·2	+1·5
53·4	27·0	+0·6
54·6	27·2	−0·2
45·1	22·5	*nil*
42·5	21·3	,,
47·9	24·1	+0·1
35·4	17·1	−1·8
47·2	23·1	−1·0
43·7	21·1	−1·0
45·6	23·0	+0·5

The recovery test was made by comparing one volume of blood with one volume of the same blood to which 0·5 c.c. of the 50-mg. standard stock solution had been added, and which therefore equalled 25 mg. Here also single determinations were made, and our results would have been better if we had delivered the standard from a microburette instead of a pipette. It should be noted that the recoveries represent the difference between two independent colorimetric determinations, consequently the error revealed is the product of two independent errors.

TABLE II.

Blood.	Blood with 25 mg. of Fe added.	Recovery.
21·3	45·1	23·8
24·1	49·1	25·0
17·1	41·4	24·2
23·1	46·8	23·7
23·0	47·6	24·6
21·1	47·2	26·1
19·2	44·9	25·7
24·1	49·0	24·9
24·2	49·5	25·3
22·6	48·2	25·6

The readings recorded in Tables I and II are a consecutive series, from which nothing has been omitted.

In order to investigate the relation of Fe in blood to the Hb concentration, some standard of Hb measurement must be chosen and a method of calculating the Fe in Hb must be found. The last requirement embraces some of the fundamental questions connected with Hb.

It is generally received that a molecule of Hb is composed of four hæmatin groups associated with a histone, globin, and that Hb is probably a polymeride of four hæmochromogen units formed with globin. The globins are species-specific proteins, and to this fraction of the Hb molecule is attributed the species differences found. So far as is known there is but one hæmatin, and Roche and Bendrihem (1931) have described an interesting demonstration of its universal distribution. They isolated hæmatin from snail bile, from the cytochrome of yeast, and from cytochrome C. The hæmatin from each source was combined with globin prepared from horse Hb, and the resulting pigments were found to be spectroscopically identical and to give the usual derivatives, O-Hb, CO-Hb and met-Hb in each case. The composition of hæmatin is still under discussion concerning some details of its graphic formula, but it is agreed that it contains one atom of Fe, and that Hb can take up and release one molecule of oxygen for each of its hæmatin groups. It follows that the oxygen capacity of Hb can be calculated from its iron content.

It is customary to speak of Hb as if it were one universal substance; actually there are probably as many hæmoglobins as there are species whose blood contains it. It has been shown that the crystallographic characters of hæmoglobin obtained from different species differ widely. Barcroft and

Barcroft (1923) have demonstrated striking differences in the gas affinities of various hæmoglobins. It has also been shown by a number of workers that the wave-lengths of the α and β bands are not identical in all species.

In the face of these facts one is forced to conclude that the hæmoglobins have different chemical structures, and it follows that an unproven assumption of identical integral photo-transmission should be treated with reserve. This point was tested with the Haldane Hb-meter, which is said to be prepared from ox blood. A number of Haldane standards were opened and compared by various methods in a colorimeter with human blood. As a result of what was found the following formal investigation was made :

Four Haldane standards were opened, gassed, and centrifugalized at 3000 r.p.m. for 20 minutes. Perfectly transparent solutions resulted. The solutions were then diluted to 1 in 200 with an equal volume of distilled water. Similarly 10 samples of human blood were made into 1 in 200 solutions of CO-Hb. The human solutions and the standards were then compared in a colorimeter which had received stringent optical adjustment. Next all the standards and human solutions were converted to acid hæmatin by the addition of an equal volume of $N/5$ HCl. They were found to give remarkably transparent solutions, quite free from the gross turbidity which is formed by the ordinary method of converting blood to acid hæmatin. Close inspection under critical illumination showed that the standards were, if anything, very slightly turbid compared with the human solutions. If such was the case it was an error in the right direction.

The human solutions reached full colour development in 15 hours, but the standards required 36 hours at room temperature, after which all remained constant for 5 days. They were not tested beyond that period.

When full development had been reached, another colorimetric examination was made to determine the relative concentration of acid hæmatin. It is to be observed that any inequalities in the concentration of different standards—and they were found to exist—does not vitiate this experiment, because it is a purely relative comparison of CO-Hb colour intensity and of acid hæmatin colour intensity.

TABLE III.—*Ten Samples of Human Blood Compared with the Mean of Four Haldane Standards.*

	As CO-Hb.	As acid hæmatin.
	97 p.c.	109 p.c.
	109 ,,	124 ,,
	81 ,,	91 ,,
	89 ,,	102 ,,
	88 ,,	97 ,,
	94 ,,	101 ,,
	89 ,,	98 ,,
	103 ,,	108 ,,
	106 ,,	116 ,,
	90 ,,	101 ,,
Mean	94·6 p.c.	104·7 p.c.

The differences found are large enough to be significant, and it is evident that the colour match as CO-Hb is not a guide to the comparative concentrations of acid hæmatin. It is known that there are many hæmoglobins, whereas there is only one hæmatin, and on that ground the reading obtained by the acid hæmatin method is to be preferred. We offer no discussion on the causes of the difference, but would point out that whilst a vast amount of work has been done on the ultra-violet absorption of Hb, it is almost impossible to obtain information from the literature on the transmission in the visual spectrum. The " general " absorption has been neglected, although the use of an animal Hb standard for comparison with human Hb presupposes that the general absorption is identical for all animals.

The Newcomer (1919, 1923) method of estimating Hb concentration was finally chosen for this work because it gives the highest Hb readings, the disc is calibrated spectrophotometrically by gas analysis of human Hb, and most of the previous work has been done with it. Readings were also taken by the Haldane method, with a standard which subsequent experience showed to be reading about 5 p.c. higher than the mean standard in the colorimeter.

In order to calculate the concentration of Hb from the Fe concentration it is necessary to know approximately the molecular weight of Hb. For many years this has been taken to be 66,700, but modern work tends to show that the value is higher. Barcroft (1928) states that " the least possible molecular weight of hæmoglobin is $(16{,}700)_n$ ". If $n = 4$, then Hb is 66,800 as a minimum. Adair (1925), working at the Low Temperature Research Station, Cambridge, examined the osmotic pressures of the Hb of man, horse, ox and sheep ; he deduced a molecular weight of 67,000, or " about four times " the equivalent (16,700) as determined by iron analyses. Apparently Adair found no difference in molecular weights of the four kinds of Hb. Svedberg and Nichols (1927) calculated the molecular weight from the rate of sedimentation in a special high-speed centrifuge and found it to be 68,000. Northrop and Anson (1929) employed the diffusion co-efficient with Einstein's equation (1908) and deduced 68,500, $\pm$ 1000, for CO-Hb (bovine).

The preparation of pure crystalline human Hb has not yet been confirmed, and data obtained from dry amorphous powders of possibly uncertain composition may not be reliable, although it must be admitted that some of the methods for the preparation of these powders appear to leave little room for gross impurities. The position therefore is that the hæmoglobins will probably be found to show only small differences in their molecular weights, and not in any way comparable to the differences found amongst the hæmocyanins (Svedberg, 1929).

Since Adair was unable to find any measurable difference in the four species examined, it appears that 68,000 is a medium value according to the combined information at present available.

It should be mentioned that Williamson (1916) describes a method of preparing crystalline human Hb, but the character of the crystals is not given ; the preparation was used as the standard in his extensive spectrophotometric determination of the human normals, and it is one of the few determinations which was based upon weight of Hb instead of the oxygen capacity method with subsequent calculation.

The oxygen capacity method of determining the concentration of Hb is derived from the conception that each hæmochromogen group in Hb is capable of taking up and releasing one molecule of oxygen, and it should therefore be possible to deduce the iron and exchangeable oxygen content of Hb from each other, if one is known exactly. The value of the iron estimation is the subject of this paper, but the oxygen capacity is the only method of primary measurement in common use. The ferricyanide reaction is considered to evolve the exchangeable oxygen in a volume strictly proportional to the Hb concentration. This assumption is, in fact, not correct, and a discrepancy has been demonstrated by Jenkins and Don (1931), who showed that more oxygen is evolved proportionally in weak concentrations of Hb. Recently Hill and Holden (1927) have shown that ferric tartrate can be substituted for ferricyanide, and it is possible that better proportional results will be obtained.

But in view of the infallibility and simplicity which are usually attributed to the ferricyanide reaction, it may be of some service to point out certain aspects of the reaction. Essentially it consists in the release of the molecular exchangeable oxygen from the oxy-Hb and the oxidation of the reduced Hb so formed to the true oxide of Hb, which is met-Hb. This must involve a supply of oxygen from a second and unknown source and the initiating agent of the reaction is also unknown. The latter may be a superoxide normally present in blood with its action accelerated by the addition of an ionizing iron salt, in which case a small proportion of the molecular oxygen may be taken up by this hypothetical superoxide. But the bulk of the oxygen with a sufficiently high oxidation potential to form met-Hb is probably derived from the splitting of a water molecule, in which case the fate of the hydrogen released comes into question. The nascent hydrogen might possibly react with some of the molecular oxygen, or it might reduce potassium ferricyanide to potassium ferrocyanide with the formation of a molecule of hydroferrocyanide acid ; the latter is a strong acid which would immediately decompose any inorganic carbonates present. Without pursuing the subject further we think that enough has been said to show that the ferricyanide method of determining the oxygen capacity depends upon an obscure and complicated series of reactions, and that the results obtained are not necessarily to be accepted at their face values.

For purposes of calculation the following data are used in this work :

Atomic weight of Fe	55·84
,, ,, oxygen	16·00
Molecular weight of Hb	68,000

From the above and from the assumed structure of Hb already mentioned there is 223·36 g. of Fe in 68,000 g. of Hb, which is 0·328 p.c. The more widely quoted value of 0·335 p.c. must be deduced from an Hb molecular weight of 66,700.

The method of calculating the distribution is to determine the whole blood Fe (W.B. Fe), subtract the plasma Fe, and calculate the Fe contained as Hb from a Newcomer Hb estimation. The total cellular iron (T.C. Fe) is the W.B. Fe minus the plasma Fe. The non-Hb cellular iron is the T.C. Fe

minus the Hb Fe. The non-Hb cellular iron (N.H.C. Fe) is expressed as a percentage of the T.C. Fe.

The W.B. Fe determinations are stated to 0·1 mg., which means a significance of 1 part in 500. It is not claimed that such accuracy exists, and it is probable that the method is accurate for duplicate determinations to about 1 part in 200, as the colour developed is strong and easy to match. The Hb estimations are probably subject to the usual error of about 2 p.c. Consequently grand means are not stated to lengths which are merely excursions in arithmetic.

RESULTS.

No attempt was made to determine the Hb concentration of normal males, as we consider that that has already been made sufficiently clear. The specimens were collected partly from normal volunteers, but mostly from hospital patients awaiting minor operations, or similar cases in which there was no obvious ground for supposing that the patient was anæmic. It will be seen that most of them are in fact slightly subnormal, but no fundamental blood change was present. The cases are arranged in order of whole blood Fe concentration, and as some arbitrary level for the normal must be selected, we have taken 50 mg. of W.B. Fe as the minimum.

TABLE IV.—*Normal Males.*

T.W.B. Fe.			T.C. Fe.		
Duplicates.		Mean.	Hb Fe.	Non-Hb Fe.	Percentage of F. to T.C. Fe.
A.	B.	C.	D.	F.	
60·5	60·5	60·5	55·6	4·6	7·7
59·8	59·4	59·6	53·4	5·9	9·9
56·8	57·4	57·1	52·6	4·5	7·9
55·9	56·3	56·1	50·3	5·8	9·5
54·8	55·1	55·0	50·2	4·5	8·2
54·7	55·0	54·9	51·3	3·3	6·1
55·2	54·4	54·8	50·9	3·6	6·6
54·5	55·1	54·8	49·5	5·0	9·2
54·8	54·4	54·6	49·8	4·5	9·0
54·4	54·4	54·4	50·9	3·2	5·9
54·2	54·2	54·2	48·5	5·4	10·0
53·6	54·6	54·1	49·8	4·3	8·6
54·1	54·1	54·1	50·1	3·7	6·9
54·2	53·4	53·8	46·8	6·7	12·3
52·9	53·7	53·3	50·0	3·0	5·7
52·9	53·3	53·1	48·6	4·2	7·9
53·2	52·9	53·1	50·0	2·8	5·5
52·3	52·9	52·6	47·8	4·5	8·6
51·9	51·9	51·9	45·6	6·0	11·5
52·0	51·5	51·8	47·5	4·0	7·8
51·6	52·0	51·8	47·2	4·3	8·3
51·4	52·0	51·7	48·2	3·2	6·2

TABLE IV.—(*cont.*).

T.W.B. Fe.			T.C. Fe.		
Duplicates.		Mean.	Hb Fe.	Non-Hb Fe.	Percentage of F. to T.C. Fe.
A.	B.	C.	D.		
51·2	51·1	51·1	46·0	4·8	9·4
50·0	50·0	50·0	46·7	3·0	6·1
49·7	49·7	49·7	44·5	4·5	9·9
48·7	48·7	48·7	44·2	4·2	8·7
48·4	49·0	48·7	44·3	4·1	8·5
48·2	48·4	48·3	44·7	3·3	6·9
48·2	48·2	48·2	45·9	2·0	4·2
47·5	47·5	47·5	43·9	3·3	7·0
47·2	47·2	47·2	43·0	3·9	8·5
47·0	47·2	47·1	41·9	4·9	10·5
45·7	45·9	45·8	44·1	1·7	3·9
45·4	45·8	45·6	42·0	3·3	7·3
45·6	45·4	45·5	42·1	3·1	6·9
42·2	42·8	42·5	38·9	3·3	7·8
41·1	40·8	41·0	36·7	4·0	9·9

Mean Values of Table IV.

W.B. Fe of 50 mg. or more : 24 cases.

W.B. Fe	53·4
Hæmoglobin Fe	49·5
Non-Hb cellular Fe	3·6
Percentage of N.H.C. Fe to T.C. Fe	6·8

W.B. Fe below 50 mg. : 13 cases.

W.B. Fe	46·3
Hæmoglobin Fe	42·8
Non-Hb cellular Fe	3·5
Percentage of N.H.C. Fe to T.C. Fe	7·5

There is no significant difference between the two groups, and they show that the proportion of N.H.C. Fe to the T.C. Fe is about 7 p.c. when the Newcomer disc is used.

No examination of young children or of women past the menopause was made, but a few young nurses were investigated four or five times each at 7-day intervals, thus covering a menstrual cycle. We desire to record our thanks to them for permitting the examinations. The data collected are rather cumbersome ; the record of each individual is chronologically arranged, so that the first set of readings are those which immediately follow the cessation of menstruation.

All the W.B. Fe determinations were done in duplicate.

TABLE V.—*Menstrual Cycle of Young Women.*
(28–29 days in every case.)

		Hb Fe.	N.H.C. Fe p.c.	Plasma Fe.
Case 1 .	1st	47·0	8·3	0·4
	2nd	43·0	7·5	0·6
	3rd	42·4	10·5	0·8
	4th	42·4	9·5	0·9
,, 2 .	1st	41·3	8·3	0·5
	2nd	42·3	8·7	1·0
	3rd	44·8	14·5	1·2
	4th	44·5	11·1	1·1
,, 3 .	1st	42·0	9·5	0·4
	2nd	41·0	6·4	0·7
	3rd	39·8	9·7	1·1
	4th	38·5	14·0	0·9
,, 4 .	1st	42·4	8·5	0·4
	2nd	42·1	9·3	0·8
	3rd	42·1	11·2	1·2
	4th	41·7	9·2	0·6
,, 5 .	1st	43·5	3·5	0·4
	2nd	43·8	8·5	0·9
	3rd	Specimen spoilt.		
	4th	39·6	11·5	1·2
,, 6 .	1st	44·9	4·6	0·9
	2nd	Specimen spoilt.		
	3rd	42·5	9·8	1·2
	4th	43·2	8·5	0·5
,, 7 .	1st	40·6	10·4	1·1
	2nd	39·4	14·1	0·9
	3rd	39·9	12·2	0·9
	4th	39·6	14·8	1·0
,, 8 .	1st	42·6	6·9	0·9
	2nd	41·4	7·5	1·0
	3rd	40·3	11·5	0·7
	4th	40·3	9·6	1·2
,, 9 .	1st	41·8	5·8	1·0
	2nd	41·1	5·7	0·8
	3rd	41·5	14·5	1·0
	4th	42·4	9·5	1·6

It will be observed that all these young women have Hb concentrations below the normal mean, although they considered themselves to be in good health. Four were on night duty, and some of the others had not yet had their annual holiday.

As this work must of necessity touch the question of the feasibility of calculating the Hb Fe concentration from that of the whole-blood iron, we think it will be informative to append a short selection of various forms of anæmia which have come under our notice :

TABLE VI.

Diagnosis.	Hb Fe.	N.H.C. Fe p.c.	Plasma Fe.
Pernicious . . .	13·8	26·9	5·8
,, . . .	13·5	30·0	1·7
,, . . .	30·2	18·0	3·8
,, (treated) . .	53·5	10·5	0·4
Aplastic . . .	10·2	38·0	1·6
Uterine hæmorrhages .	14·9	24·0	0·8
Not diagnosed . .	24·9	32·0	0·5
Microcytic . . .	19·5	15·9	1·1
,, . . .	20·1	10·0	1·1
Post-puerperal . .	16·0	16·0	0·6
Milk diet, 1 month . .	41·5	20·0	0·7
Idiopathic subchromic .	32·1	20·0	0·4
,, ,, . .	14·9	13·0	1·6
,, ,, . .	19·5	20·0	1·1
,, ,, . .	21·1	5·9	1·0
Megalocytic, gastric carcinoma	8·5	19·0	0·8

DISCUSSION.

The method of iron analysis described confirms by chemical analysis the spectrophotometric investigations of Barkan and of Winegarden and Borsook. Agreement is good both for plasma iron and non-Hb cellular iron. In addition we have been able to show that women have more plasma iron than men; it has been suggested that this is due to increased requirements for blood regeneration incidental to menstruation.

As far as our results permit one to judge there appears to be some ground for the suggestion, and if any experimental significance can be attached to the weekly variations in the plasma-iron concentration, it seems to show that the concentration is at its height before and during menstruation, and falls to its lowest level immediately after menstruation has ceased. This might be taken to indicate that the rate of regeneration of blood in the young female is in part influenced by the menstrual cycle.

The significance of the N.H.C. Fe is obviously debatable. Two basic conceptions of its origin are, firstly, that the iron is included in the cell during its production in the marrow or, secondly, that it is evolved during the breaking down of Hb in the functional life of the erythrocyte.

The findings recorded in the group of anæmic cases offer some evidence that can be adduced in the discussion. The N.H.C. Fe is large in all types of severe anæmia, including those which might be expected to respond to treatment with iron. It is difficult to perceive why the hæmopoietic system should include in a new erythrocyte iron other than in the form of Hb when the organism is then suffering from an urgent need of iron, and further, why the N.H.C. Fe should be proportionally higher in severe anæmia than in the milder forms.

If, on the other hand, it is supposed that this Fe represents a breakdown

product derived from ageing Hb, then its relative increase in severe anæmia can be explained as a compensatory attempt to prolong the life of the individual erythrocyte beyond that period at which it would be normally removed from the circulation. This hypothesis obtains some small support from the N.H.C. Fe readings in the normal females, if it be granted that blood regeneration is influenced by the menstrual cycle. The N.H.C. Fe rises steadily during the intermenstrual period, and might be taken to indicate an increasing proportion of aged cells which are largely removed during the active phase immediately after menstruation.

In connection with foregoing speculation it is of interest to recall that Escobar and Baldwin (1934) concluded from oxygen tension studies that the life of an erythrocyte in man is 18 to 30 days. At the same time it must be borne in mind that examination of the blood as a whole represents the findings for the total erythrocytic population, and any inferences must be applied with caution to the metabolism of a given individual cell. In males there is no reason to suppose that a cyclical fluctuation in the mean age of the erythrocytes exists, so that if the mean N.H.C. Fe is 7 p.c., it can be inferred that a cell is normally removed from the circulation when the N.H.C. Fe reaches 14 p.c., always assuming that new cells contain no N.H.C. Fe.

The general proposition that the Hb concentration can be calculated directly from a determination of the whole-blood iron rests upon a fallacious assumption, and any apparent agreement which has been obtained is fortuitous, and due to large errors in the technique employed.

The proportions of N.H.C. Fe and of plasma Fe have been shown to differ in males, in females, and in the anæmias. In the latter there is no means of making an empirical allowance which would have any claims to accuracy. Hitherto the plasma Fe has been regarded as insignificant, but in the course of a preliminary study of the anæmias we have already been able to establish that the plasma Fe varies significantly, and can be much influenced by various forms of treatment.

It is possible that chemical separation of N.H.C. Fe might enable one to calculate the Hb concentration from the remaining cellular Fe, and we found in a short series of examples that the N.H.C. Fe as determined by our method was in surprisingly good agreement with that determined by the method of dialysis employed by Winegarden and Borsook. The colour of the dialysate precludes any possibility of its contained Fe being acid hæmatin. But a more extensive series of observations is necessary before any definite conclusions can be drawn.

SUMMARY.

1. A colorimetric method of determining the distribution of iron in blood is described.

2. It is shown that the total Fe in blood is greater than can be accounted for by any known method of estimating the Hb concentration.

3. The observations of Barkan, confirmed by Winegarden and Borsook, are now further confirmed.

4. The average non-Hb cellular Fe is normally about 7 p.c.

5. The plasma Fe in males is about 0·3 mg. per 100 c.c. of whole blood, and about 0·8 mg. in young women.

6. Preliminary work shows that in the anæmias the non-Hb cellular Fe and the plasma Fe differ largely and significantly from the normal.

7. The estimation of whole blood Fe as a means for calculating the Hb concentration is valueless, and the instances of apparent agreement are due to large errors in the methods of determining the Fe concentration.

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OXYGEN POISONING AND TUMOUR GROWTH.

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MANY experiments have been conducted to determine the effects of variations in pressure itself, and in percentage of oxygen in the inspired air upon cancer growth. Warburg and his collaborators (1927) killed the bulk of the cancer-cells in rats bearing implanted tumours by keeping the rats for 40 hours in an atmosphere containing the minimum amount of oxygen (5 volumes p.c. of 1 atmosphere) tolerated by the animals for that duration of time. However, the cells at the periphery of the tumours still remained alive and continued the growth of the tumour. These results were confirmed by Campbell and Cramer (1928).

In contrast with these experiments with low pressure, we have the recent research of Basset and his co-workers (1935), who found that a pressure of 1800 atmospheres for half an hour destroyed mouse sarcoma 37 suspended in physiological saline, but a pressure of 1000 atmospheres for the same duration failed to do so. In these experiments they obtained some interesting evidence that the Rous sarcoma, a virus sarcoma, behaves like some viruses, *e. g.* vaccinia virus, in being more resistant to the increased pressure than the mouse tumours, which are apparently non-virus tumours that is, concerned with malignant cells. Such pressures are, of course, much too high for *in vivo* treatment with oxygen gas. Fischer and Anderson (1926), using much lower pressures, observed that the fibroblasts of the fowl, exposed in a culture medium of fowl plasma and freshly-prepared embryonic tissue juice, were killed in 10·5 hours by 7 atmospheres of oxygen, while Rous fowl sarcoma-cells were destroyed in 9·5 hours. They also found that with only 1 atmosphere pressure of oxygen, the sarcoma-cells were killed in 26·5 hours, while the fibroblasts remained alive for 33 hours. Fischer and his co-workers (1927), therefore, exposed tumour-bearing mice to 1·6 to 2 atmospheres of pure oxygen for 18 to 24 hours, and succeeded in bringing about a disappearance of the tumour in a considerable number of animals, but only if treatment were applied during the first week of transplantation. It is well known that such pressures of oxygen for such durations of time may cause pneumonia (Lorrain Smith, 1899, and others), and pneumonia frequently occurred in the animals treated by Fischer. To avoid pneumonia Campbell and Cramer (1928) used only 60 p.c. of an atmosphere of oxygen, but used it almost continuously for 2 weeks, employing rats and mice with transplanted tumours ; such exposures had no effects upon the rate of growth of the tumours.

More recently de Almeida (1934*a*) discovered that if he starved rats for 2 to 3 days, they were rendered more resistant to the poisonous effects of the

oxygen; we have now confirmed this result for starvation (see Table IIA). de Almeida (1934*b*) used up to 6 atmospheres' pressure of oxygen for 2 hours, and claims regressions in 23 p.c. of his starved animals in one experiment. Such experiments have not been repeated, so far as we know, on cancer patients during starvation, but Behnke and his colleagues (1935) have found that normal man can breathe pure oxygen with comparative safety as follows: 4 hours at 1 atmosphere, 3 hours at 2 atmospheres and 2 hours at 3 atmospheres; also he can breathe oxygen at 3 atmospheres for 3 hours without distressing symptoms, but at 4 atmospheres convulsions occurred in one subject and syncope in another after exposures of approximately 45 minutes. These facts will limit the use of de Almeida's therapy in a human patient, but it is likely that starvation for a couple of days may increase the patient's resistance to oxygen poisoning provided he is strong enough to tolerate starvation. Sir Henry Dale drew our attention to this recent work of de Almeida, and we now report some experiments of a similar nature.

TECHNIQUE.

Tumours used.—de Almeida used Rollo's spindle-cell rat tumour, and we have employed the Walker rat-tumour, mouse carcinoma No. 63 (Bashford's tumour) and the Twort mouse-tumour. We used white rats weighing about 60 to 80 g. and mice about 20 g. Rats of the above weight do not tolerate starvation well beyond about 2 days, nor do the mice beyond about 29 hours. We found also that our starved rats did not tolerate 6 atmospheres of oxygen for one hour without a fairly high death-rate, so we used 5 atmospheres for one hour in most experiments, and even then there were usually one or two deaths out of a total of every 10 animals exposed. The mice only tolerated 4 atmospheres for one hour, and this always proved too much for one or two of the dozen or so unacclimatized mice exposed at the same time; such results have been observed by others (*e. g.* L. Hill, 1912).

The tumour tissue was macerated in the usual way with scissors, and about 1/40th of a c.c. was inoculated into each rat, and about 1/60th of a c.c. into each mouse.

Apparatus.—The experiments were carried out in a small compression chamber at the premises of Messrs. Siebe Gorman & Co., 187, Westminster Bridge Road, London. The interior of this cylindrical chamber is 47 cm. in length and 22·8 cm. in diameter. The animals were enclosed in five separate compartments of a long narrow wire cage, 2 rats or up to 6 mice in each compartment. On each side of the outside of the cage a long narrow container with soda lime was placed to absorb carbon dioxide and water vapour. Also to prevent the concentration of the carbon dioxide rising, a continuous through-ventilation of the chamber with oxygen was maintained. Samples of gas from inside the chamber were analysed in each experiment, and carbon dioxide was present only in minute traces, while the oxygen percentage was always above 98·5. It has been proved that oxygen poisoning—if not due to—is accelerated by a high pressure of carbon dioxide in the tissues (Gesell, 1923; Campbell, 1930; L. Hill, 1933, and others); therefore great care was taken to prevent accumulation of carbon dioxide in the chamber.

Decompression.—In order to prevent bubble formation in the tissues by too rapid decompression, the routines shown in Table I were followed. More rapid decompression was tried, but was not so satisfactory.

TABLE I.—*Showing Rates of Decompression.*

Routine for rats.		Routine for mice.	
Minutes.	Condition.	Minutes.	Condition.
0–60	Exposed to + 60 lb. O_2	0–60	Exposed to + 45 lb. O_2
60	Start to decompress from + 60 lb.	60	Start to decompress from + 45 lb.
63	Pressure at + 40 lb.	63	Pressure at + 30 lb.
69	„ + 30 lb	66	„ + 25 lb.
74	„ + 20 lb.	71	„ + 20 lb.
79	„ + 15 lb.	76	„ + 15 lb.
84	„ + 10 lb.	81	„ + 10 lb.
89	„ + 5 lb.	86	„ + 5 lb.
90	„ + 0 lb.	90	„ + 0 lb.

External temperature.—P. Bert (1878) observed that one of the symptoms in oxygen poisoning was a fall of body temperature, sometimes by as much as 10° C. This has often been confirmed. de Almeida (1934) records rectal temperatures of 26°, 24° and 22° C. when the external temperature was 20° C. It occurred to us that this great fall of body temperature might be protective; and we found that if the body temperature is prevented from falling greatly by keeping the external temperature between 31° and 35° C. the poisoning may be greatly enhanced. We exposed normal white rats to 6 atmospheres of oxygen for 30 minutes and decompressed them slowly for 20 minutes; when the external temperature was kept at about 33° C. there were rarely any survivors, but at 24° C. (21–27° C.) there were rarely any deaths (Table IIA, Group 4). Observations of longer duration give similar but smaller differences. From this it seems likely that oxygen poisoning concerns the combustion of some substance, which takes place more rapidly at 33° C. than at 24° C. The absolute oxygen pressure is a factor, since at 4 atmospheres pressure of oxygen for 30 minutes at 33° C. all the rats survive. Also white rats easily tolerate 37° C. at least for some hours in ordinary air. In our experiments with the tumour rats and mice we kept the external temperature between 21 and 27° C.

TABLE IIA.—*Effect of External Temperature on Oxygen Poisoning* (*Normal White Rats*).

Experimental group number.	Pressure O_2 atmospheres.	Duration of compression (minutes).	Time for decompression (minutes).	Starved (S.) or non-starved (N.S.).	Survivors at external temperature. 33° C.	Survivors at external temperature. 24° C.
1	5	60	30	S.	0 out of 5	10 out of 10
				N.S.	0 „ 5	4 „ 5
2	6	60	36	S.	..	4 „ 12
				N.S.	..	1 „ 12
3	6	40	20	S.	0 „ 3	3 „ 3
				N.S.	0 „ 6	1 „ 6
4	6	30	20	S.	3 „ 10	13 „ 15
				N.S.	0 „ 10	14 „ 15
Total survivors				S.	3 „ 18	30 „ 40
				N.S.	0 „ 21	20 „ 38

TABLE IIB.—*Decompression Routine for Experimental Group No. 4.*

Minutes.		Condition.
0–30	.	Exposed to + 75 lb. O_2.
32	.	Pressure at + 60 lb.
34	.	„ + 50 lb.
36	.	„ + 35 lb.
40	.	„ + 25 lb.
44	.	„ + 15 lb.
48	.	„ + 7 lb.
50	.	„ + 0 lb.

Temperatures below 20° C. are not suitable in these experiments, particularly with mice, because during decompression they may become wet and chilled. Temperature was controlled by passing steam through a coiled copper tube within the chamber.

RATE OF TUMOUR GROWTH.

The Walker rat-tumour.—Eighteen rats were inoculated with minced tumour substance, and 3 days later 10 of them, after 2 days' starvation, were exposed for 60 minutes to 5 atmospheres of oxygen and then decompressed for 30 minutes, as shown in Table I. Once a week for 7 weeks they were similarly starved for 2 days and exposed to high oxygen pressure. These 7 exposures had no retarding effect upon the rate of growth compared with the rate of growth of the tumour in the controls (Table III). As a matter of fact, growth was rather more rapid in the experimental group. The length, breadth and depth of the tumours were measured weekly in millimetres, and their volumes in cubic centimetres were calculated from these figures. After the death of the animals macroscopic and microscopic examinations of all the tumours were carried out, and there was no difference between the experimental and control tumours ; in both cases there was always at least half of the tumour in a more or less necrosed condition.

Mouse tumours.—When the Twort mouse tumours had grown for 2 weeks, the experimental mice were exposed to 4 atmospheres of oxygen for 1 hour once a week for 7 weeks without any effect upon the rate of growth of the transplanted tumours. In this experiment the control tumours at the start were much smaller than the experimental tumours, the larger tumours being chosen on purpose for oxygen exposure, since any retardation would be more easily recognized. After allowing for this difference in size at the start, there was no difference eventually in rate of growth ; this is seen clearly if the results be plotted out.

The mice with the Bashford tumour were exposed to high oxygen pressure for the first time about 11 days after inoculation. They were given 5 exposures to 4 atmospheres of oxygen, usually one exposure a week. As with the rats, the usual starvation period prevented more frequent exposure than this. The tumours in the experimental group (Table III) grew somewhat more rapidly than those in the control group.

Numerous exposures of a mouse, with a spontaneous mammary carcinoma, to similar pressures of oxygen failed to prevent the tumour growing steadily to a large size and eventually killing the animal.

TABLE III.—*Details of Rate of Growth of Tumours.*

Tumour.	Number of animals.	Size of tumours, c.c.; in brackets, number of survivors. 2nd week.	3rd week.	4th week.	5th week.	6th week.	7th week.	8th week.	9th week.
Walker, rat	10 exposed to O_2	..	0·36 (10)	2·92 (10)	21·26 (7)	43·99 (3)	54·20 (2)	..	..
	8 controls	..	0·40 (8)	2·93 (8)	18·09 (6)	36·60 (4)	45·80 (3)	..	..
Twort, mouse	6 exposed to O_2	0·31 (6)	2·48 (6)	8·29 (6)	12·24 (6)	13·96 (6)	19·67 (6)	31·15 (6)	..
	6 controls	0·04 (6)	0·59 (6)	2·71 (6)	3·72 (6)	5·56 (6)	10·82 (6)	12·23 (6)	19·63 (6)
Bashford, mouse	15 exposed to O_2	..	2·18 (15)	6·17 (9)	..	35·25 (2)	..	..	..
	13 controls	..	2·37 (13)	..	12·00 (9)	30·96 (6)	..	..	..

TABLE IV.—*Blood-Sugar Estimations, g. per 100 c.c.*

	Control rats at 24° C.		Compression rats at 24° C.		Compression rats at 33° C.	
	9 non-starved.	9 starved.	9 non-starved.	9 starved.	9 non-starved.	9 starved.
Average blood sugar	0·114	0·073	0·109	0·044	0·144	0·061
Limits of blood sugar	0·074–0·155	0·040–0·090	0·039–0·186	0·027–0·074	0·106–0·231	0·031–0·090

DISCUSSION.

Thus we failed to repeat de Almeida's destruction of tumours by oxygen. We do not know the reason ; he used a different tumour, and also some of his rats seem to have been more resistant to the high pressure of oxygen and starvation, since they were able to stand rather more drastic treatment than ours. He may have decompressed his animals more rapidly and thus freed bubbles of oxygen in the tumour tissue. It is difficult to see why one or two hours' exposure to 6 atmospheres of oxygen should destroy the tumour *in vivo* when much longer durations seem necessary *in vitro*. We carried out experiments *in vitro*, exposing macerated Walker rat-tumours to 9 atmospheres of oxygen for 35 minutes and to lower pressures for a total of 60 minutes ; but the tumours resulting from inoculation grew just as vigorously as those of controls. Previous observers, as already stated, have used higher pressures *in vitro* without any effect on the vitality of the tumour-cells. de Almeida's results may have been due to an indirect effect on the tumour through some action on the host.

As stated above, we were able to confirm his finding that animals starved for a day or two are more resistant to high oxygen pressure than are fed animals. We attempted to determine the reason. Dr. F. G. Young kindly estimated the blood-sugar in the starved and fed animals, and as a rule the former had a much lower blood-sugar (Table IV). On two occasions only a very low blood-sugar (0·039 g.) was found in " fed " rats, but this may have been due to these rats not really feeding, although food was placed in their boxes. The low blood-sugar might explain why starved rats are more resistant to high oxygen, if oxidation of glucose is concerned in any way with the poisoning produced by the high oxygen pressure ; but injections of glucose do not appear to have any great influence upon the result. In some of the experiments a mild and transient glycosuria occurred ; this has been noted by others (Auler, 1927). The high oxygen pressure did not appear to influence the blood-sugar to any great extent, nor did the external temperature.

The external temperature does, however, greatly influence the oxygen poisoning, as we have demonstrated for the first time. It seems that some combustion is going on more vigorously at 33° C. than at 24° C. Recently Barsoum and Gaddum (1936) have observed that histamine is increased in the blood of patients suffering from superficial burns, these, of course, being due to high temperature and oxygen pressure at 21 p.c. of an atmosphere. This led us to the suggestion that very high oxygen pressure (400–600 p.c.) at 33° C. might lead to a similar " burning "—of some tissue, *e. g.* lung—with production of histamine. Dr. C. Code kindly estimated the histamine in normal control rats and found about 0·4 to 0·5γ per c.c. of blood ; in one group of rats exposed to 5 atmospheres' pressure of oxygen he found nearly three times as much, namely, 1·27γ per c.c. All the rats were killed in a similar way with chloroform and the blood collected at once from the heart. This aspect of the research is still under investigation ; histamine itself does not appear to be responsible, since injections of histamine do not influence oxygen poisoning to any extent ; nevertheless, some substance closely related to histamine may be responsible. Substances, which administered subcutaneously have been

found to enhance oxygen poisoning in white rats (80 g.), are thyroxine (0·4 mg.), dinitrophenol (2 mg.), *ac*-tetrahydro-β-naphthylamine (0·5 c.c., 1 p.c.), adrenaline (0·02 mg.), pituitary extract (above 3·5 units, posterior lobe), insulin (0·025 unit) and eserine (0·045 mg. administered with atropine 0·075 mg.). Discussion of these results will follow later.

SUMMARY.

1. de Almeida's destruction of Rollo's tumour in living rats with high oxygen pressure could not be confirmed with the different tumours (rat and mouse) used in the present work.

2. His finding that starved rats tolerate high oxygen pressure better than do fed rats is confirmed.

3. It is shown for the first time that external temperature may exert an effect on oxygen poisoning. Exposing white rats to 6 atmospheres of oxygen for 30 minutes at 33° C. usually results in death ; at 24° C. it rarely results in death. The fall (by 10° C.) of body temperature observed in oxygen poisoning is thus protective.

I am indebted to Sir Robert Davis for permission to use the compression chamber at Messrs. Siebe Gorman's, S.E. 1, and also to Mr. C. Pergande for much help throughout this research, and to Drs. F. G. Young and C. Code for some chemical estimations.

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AN INVESTIGATION OF THE ALLEGED TUMOUR-PRODUCING PROPERTIES OF LIPOID MATERIAL EXTRACTED FROM ROUS SARCOMA DESICCATES.

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THE importance of demonstrating the connection between the filterable tumours of fowls and those obtained by the injection of tar and its derivatives has long been recognized. Certain experiments recently reported by Jobling and Sproul (1936) appear to throw new light on this problem, and the conclusions to which their results lead would, if confirmed, represent a big advance in the study of the causation of malignant disease. Impressed with the fact that Claude (1935) had found an appreciable amount of lipoid material in purified extracts of the Rous No. 1 sarcoma, these workers proceeded to determine the tumour-producing activity of substances extracted from that tumour by means of various solvents. In the first method which they describe, fresh tumour tissue was extracted with acetone. After clarification by centrifugation and filtration this acetone fraction was concentrated *in vacuo* under nitrogen at 37° C. until only the watery residue remained. Chickens were given repeated inoculations of this watery residue at weekly intervals. The majority of the birds developed, after an interval of 3 to 4 months, tumours which were identical with the Rous sarcoma. No mention is made of the type of filter that was used for clarifying the acetone extract. The residue of the tumour after it had been dried and in good part freed of lipoids did not produce tumours. In later experiments the tumour was first dehydrated in the Flosdoff-Mudd lyophile apparatus, whereby a highly active desiccate was obtained. This was extracted with benzene or carbon tetrachloride in a Soxhlet extraction apparatus at 37° C. in an atmosphere of nitrogen under reduced pressure. The fatty material obtained after removal of the solvent was then tested for tumour-producing activity by animal inoculation as before. It is claimed that this material failed to produce tumours when inoculated by itself, but that it regularly gave rise to typical sarcomata if, prior to inoculation, it was mixed and inoculated with an extract of chicken inflammatory cells. The extract was prepared by inoculating kieselguhr into the pectoral muscles of normal chickens. The resulting inflammatory area, which consisted mainly of mononuclear and giant-cells, was scraped out 48 hours later and a 10 p.c. suspension of the tissue in normal saline was prepared. The lipoid material, which had been evaporated to a sticky mass was, suspended in this extract, incubated under nitrogen at 37° C. for 1 hour, and injected

immediately. Tumours were produced within 2 to 3 weeks after a single injection. The residue of dried tumour after extraction still retained some degree of activity—a fact which was assumed to be due to incomplete extraction of the lipoid. The incubation *in vitro* of the lipoid and inflammatory exudate was apparently an essential procedure, because the injection of lipoid directly into the breast muscle of chickens which had been previously inoculated with kieselguhr failed to produce tumours. From these results it was concluded that the transmissible agent of the Rous No. 1 sarcoma is a specific carcinogenic substance, probably lipoid in nature, which is activated by some factor present in extracts of chicken tissue.

Although this work of Jobling and Sproul lends support to the theory on which it is based, there are certain now well-established facts concerning the nature of the Rous tumour agent which are difficult to reconcile with their views. During recent years it has been conclusively demonstrated that the tumour agent can be deposited from cell-free extracts and filtrates by means of the high-speed centrifuge (Ledingham and Gye, 1935; Andrewes and Elford, 1935; McIntosh, 1935; Amies, 1937). A centrifugal force equivalent to 20,000 times the force of gravity will achieve this result in one hour. A lipoid substance existing as a dispersed phase in an aqueous medium would, on the contrary, rise to the surface of the column of fluid when centrifugal force was applied to it on account of its low density. The actual particle size of the Rous agent has recently been determined by Elford and Andrewes (1935, 1936), both by ultra-filtration and centrifugation. The figure of 70mμ. which they give as the diameter of the infective particle accords well with the earlier work referred to above. On this evidence, therefore, the conception of an independently existing lipoid substance as the cause of the Rous sarcoma appears untenable. On the other hand, the results of Jobling and Sproul might possibly be explained by the presence of an active lipoid substance which was loosely attached to denser particles within the tumour-cells. Extraction of the active deposit obtained by centrifugation might then yield a lipoid substance with tumour-producing properties.

The fact that desiccated Rous tumour is not inactivated by fat solvents was already known from the work of other investigators. Gye and Purdy (1931) recorded the fact that the dried tumour after extraction with perfectly dry ether was as active as the untreated desiccate, and they inferred from this that the tumour agent, whatever its nature may be, was certainly not lipoid in character. Claude (1935) has also described experiments in which desiccates of this tumour were extracted first with acetone and then with an alcohol-ether mixture. About 14 p.c. of the dried tissue was found to be soluble in fat solvents. This lipoid fraction was not tested for tumour-producing activity, but the residue left after extraction was found to be as active as the original tumour desiccate.

In view of the similarity in biological behaviour of the filterable tumour agents and the viruses, it is of interest to note that the latter also are unaffected by extraction with the usual fat solvents. Thus, Dearing (1934) has shown that desiccates of tissue infected with vaccinia are not inactivated by extraction with dry ether, acetone or alcohol. If the stability of the Rous agent to the action of benzene or carbon tetrachloride is accepted, a possible explanation

of the results obtained by Jobling and Sproul at once arises. Unless special precautions are taken, the small size of the tumour agent may readily permit it to pass out of the thimble during the process of extraction in the Soxhlet apparatus : tumour agent might in this manner be introduced into, and would form a contaminant of, the lipoid substance extracted from the desiccate. This hypothesis has been tested experimentally, and the results appear to leave no doubt that the tumour-producing activity of the lipoid material can be explained in this manner.

METHODS EMPLOYED.

The general plan of the experiments was to extract highly active Rous tumour desiccates with benzene in an atmosphere of nitrogen. The lipoid solution so obtained was divided into two fractions, one of which was either filtered or centrifuged to remove any particulate matter that might be present, while the other was left untreated as a control. These two fractions, after removal of the solvent, and also the untreated desiccate and the residue obtained after extraction, were then tested for tumour-producing activity by inoculation into young chickens. The activity of the lipoid material was tested both with and without the addition of an extract of chicken inflammatory cells.

Material.—Selected portions of rapidly-growing Rous No. 1 sarcoma were minced and then dried *in vacuo* from the frozen state over phosphorus pentoxide or sulphuric acid. The method employed, which will be described in detail in a subsequent communication, allowed the complete desiccation of 100 g. of tissue in 5 to 6 hours. The dried material was stored *in vacuo* over phosphorus pentoxide at 0° C. until required. It was used whenever possible on the following day, or at most within a few days of its preparation.

Extraction.—The dried tumour was rapidly transferred to a Whatman extraction thimble or to a thimble made from several layers of filter-paper, which was plugged with cotton-wool in such a way that the material could not be washed out of the top by the solvent. The extraction was carried out under reduced pressure with benzene (analytical reagent) in a Soxhlet apparatus* fitted throughout with ground-glass joints, which were sealed with a stiff mixture of sucrose and glycerol. Connection to the pump was made by a long glass tube attached to the top of the condenser by a ground-glass joint. A boiling stick in the evaporating flask ensured steady boiling of the solvent, while efficient condensation of the vapour was obtained by a stream of ice-cold water passing through the internal coil of the condenser.

At the commencement of the extraction the apparatus was first evacuated by a water-jet pump, and then it was filled with nitrogen from a cylinder. This process was repeated. The boiling flask was then warmed in a water-bath at 35° C. and the apparatus was evacuated until the benzene commenced to boil, when the connection to the pump was almost entirely closed. Boiling then proceeded rapidly and regularly, with only a very slight loss of solvent during an extraction of several hours. Under these conditions the solution in the flask boiled at a temperature below 30° C., while the extraction took place at about 20° C.

Filtration.—At the end of the extraction, which was usually allowed to proceed for 3 to 5 hours, the apparatus was filled with nitrogen at atmospheric pressure. The extraction chamber and condenser were removed and replaced immediately by a glass head, through which passed a stream of nitrogen, and which carried a syphon tube reaching almost to the level of the liquid in the flask. To the other end of the syphon tube was attached a narrow glass tube (the filter tube) containing a plug of cotton-wool and fitted with a tap. The filter tube could be replaced during the course of the experiment by a plaster-of-paris filter or by a tube containing a Berkefeld W filter, while a similar arrangement could be adapted for filtration through a Seitz filter in an atmosphere of nitrogen. The lower end of the filter-tube was connected to a small evaporation

* This was supplied by Messrs. Quickfit & Quartz, Ltd., London.

flask connected to the vacuum pump. This flask was immersed in a water-bath at 35° C. After the stream of nitrogen had passed through the apparatus for some minutes, the syphon tube was lowered into the liquid in the extraction flask and the liquid was slowly drawn over into the evaporation flask, the rate of flow being regulated by the tap. It was therefore possible to evaporate the liquid *in vacuo* out of contact with air.

Dispersion of the lipoid material.—In order to bring the lipoid material into intimate contact with the tissue extract the former was first emulsified with water by means of a domestic cream machine. In this manner it was possible to produce a stable emulsion readily miscible with saline or tissue extract. In a few of the experiments the fatty material was dissolved in sesame oil before emulsification. Attempts to prepare suspensions of a still greater degree of dispersion, by adding a solution of the fat in alcohol or acetone to gelatine-saline with subsequent removal of the solvent *in vacuo*, were unsuccessful owing to the fact that this procedure inactivated the material.

Preparation of tissue extract.—A young chicken was inoculated into each breast muscle with 5 ml. of a 10 p.c. suspension of sterile kieselguhr in normal saline. A wide infiltration of the muscle was secured. Two inoculations were given on successive days and the fowl was killed 24 hours later. The resulting inflammatory tissue, together with some kieselguhr and normal muscle, was removed aseptically and ground in a mortar with sand. Sufficient saline was added to give a 20 p.c. suspension. This was then lightly centrifuged to remove sand and gross debris, The extract was used immediately after it had been prepared.

Incubation and inoculation.—The lipoid emulsion was divided into two equal volumes: to one of these was added one volume of the tissue extract while the other received one volume of saline. Both the filtered and unfiltered fractions of the lipoid were treated in this manner. There were thus four different preparations to be tested, and these were inoculated one into each breast muscle and one into each leg of the same chicken. The inoculations were made in triplicate or quadruplicate in Brown Leghorn chickens aged 6 to 8 weeks. At the same time the untreated desiccate and the residue obtained after extraction were suspended in saline to give a 1 p.c. suspension by weight of the dry material. The tumour-producing activity of these suspensions was then tested by inoculating a series of dilutions of each into groups of 3 or 4 chickens. The fowls were kept under observation for 50 days. Those surviving at the end of this period were then killed and examined.

RESULTS.

The results of 9 experiments in which filtration was used are shown in Table I. These clearly demonstrate that benzene extraction had no appreciable effect upon the tumour-producing activity of the desiccates. In 4 out of these 9 experiments the unfiltered lipoid gave rise to tumours, whereas the same quantity of lipoid after filtration produced none. In one similar experiment, which is not included in this series, tumours were produced by a Seitz filtrate of the lipoid extract. It was found, however, that the filter which had been used was faulty, since it permitted the passage of vaccinia virus and of bacteria. With this one exception, which can reasonably be ignored, the results indicate that the activity of the lipoid extracts is due to contamination with particulate matter. It will be noticed that the amount of fatty material extracted in a 3 to 4 hours' period was considerably less than the figure given by Claude. Prolonged extraction, however, produced a larger yield. In Experiment 9, in which the extraction was continued for 13 hours, 8 p.c. of fat was removed. In this case also the residue was found to be as active as the original desiccate. The effect of prolonged extraction was further determined by shaking the desiccate with benzene in an atmosphere of nitrogen. The shaking was carried out mechanically for 48 hours, the solvent being changed 4 times during this period. From 21 g. of tumour desiccate 0·51 g. of lipoid material was obtained. This was redissolved in benzene and then

TABLE I.—*The Tumour-producing Activity of Lipoid Material Extracted from Rous Sarcoma Desiccates.*

Experiment number.	Weight of desiccate (g.).	Duration of extraction. (hours).	Amount of lipoid extracted.		Titre of desiccate.	Titre of residue.	Tumour production with lipoid fraction.				Type of filter used.
			g.	p.c.			Unfiltered lipoid.		Filtered lipoid.		
							+ tissue extract.	+ saline.	+ tissue extract.	+ saline.	
1	3·5	3	0·190	5·5	1/50	1/50	0/3	0/2	0/3	Not tested	Plaster of paris.
2	3·0	3½	0·240	8·0	1/125	1/25	0/4	0/4	0/4	0/4	„ „
3	4·5	4	0·175	3·9	1/125	1/125	3/3	3/3	0/3	0/3	„ „
4	9·9	4	0·600	6·0	1/125	1/125	0/3	0/3	0/3	0/3	„ „
5	10·0	5	0·570	5·7	1/25	1/125	0/4	0/4	0/4	0/4	„ „
6	5·0	3	0·150	3·0	1/125	1/125	0/4	0/4	Not tested	Not tested	..
7	19·0	4½	0·750	3·9	1/25	1/25	2/4	2/4	0/4	0/4	Berkefeld W.
8	9·0	6	0·240	2·6	1/125	1/125	2/2	2/2	0/2	0/2	Seitz.
9	5·0	13	0·400	8·0	1/500	1/500	3/3	2/3	0/3	0/3	„

The results of inoculations with lipoid are shown as a fraction in which the denominator indicates the number of fowls injected and the numerator the number which developed tumours.

divided into two equal parts, one of which was filtered through a Seitz filter, while the other was passed through cotton-wool only, to remove gross contamination. The latter fraction produced a tumour in one bird out of three which were inoculated, while the filtered fraction (amounting to 83 mg. of lipoid for each fowl) was inactive.

Additional proof of the presence of tumour desiccate in the crude lipoid extract is afforded by a further experiment in which the benzene extract was shaken with an equal volume of normal saline. The aqueous and benzene layers were separated by centrifugation, and the fatty material in the latter, after removal of the solvent, was emulsified in the usual manner. Tumours were produced by the inoculation of this aqueous layer, but the lipoid recovered from the benzene layer was inactive.

Less convincing results were obtained when centrifugation was substituted for filtration. In one out of the two experiments performed the activity of the benzene extract was considerably diminished by centrifuging it at 15,000 r.p.m. (20,000 times gravity) for 30 minutes, while the small deposit so obtained was highly active. Failure to obtain complete sedimentation can probably be explained by the action of convection currents set up in the highly volatile solvent. In the other experiment of this type the crude lipoid extract was itself inactive. It may be noted that the benzene extracts, which appeared clear to the naked eye, yielded a visible deposit on centrifugation.

Finally, a similar experiment was carried out with vaccinia virus. The skin pulp of a rabbit infected percutaneously with vaccinia was dried and extracted with benzene in the usual manner. One half of the lipoid material so obtained was filtered through a Seitz filter, while the other half was left unfiltered. Titration of the residue and desiccate by intradermal inoculation showed that benzene extraction had not appreciably affected the virus; furthermore, the unfiltered lipoid fraction produced a definite vaccinial lesion, while the filtered fraction was completely inactive.

DISCUSSION.

The experiments which have been described indicate that the activity of the lipoid material extracted from Rous No. 1 sarcomata is due to contamination with traces of the tumour desiccate, and not to the presence of a chemical substance in true solution. This explanation at once disposes of the view that a second factor present in inflammatory cell extracts is essential for tumour production. The results obtained confirm this. The effect, if any, of incubation of the lipoid material can possibly be attributed to rehydration and dispersal of the particles of tumour desiccate present in the lipoid material. The results, it will be noted, yield no fresh information about the nature of the tumour agent itself.

It is improbable that the effect of filtration can be attributed to the selective absorption of a chemical substance with carcinogenic properties. The amount of material retained in the filters after washing with the solvent was only a small fraction of that which passed through them; furthermore, it would be necessary to postulate a complete adsorption of this hypothetical substance by filters of widely different nature. The removal of the activity from unfiltered

benzene extracts by centrifugation and the transference of that activity to the aqueous phase when the extract was shaken with saline again indicates a contamination with particulate material, rather than the presence of a carcinogenic substance in true solution. This view is further supported by the contrast between the rapidity with which the tumours were produced by the benzene extracts, and the length of time taken by even the most active carcinogenic hydrocarbons to produce a malignant growth.

The residual activity of the dried tumour after extraction with solvents was attributed by Jobling and Sproul to incomplete extraction of the lipoid material. The present experiments show, however, that no appreciable diminution of activity occurred after extraction with benzene. Even after a prolonged extraction during two days, a procedure which removed over 90 p.c. of the extractable lipoid material, the re-suspended residue had a titre as high as that of the original tumour desiccate. Furthermore, no significant difference could be detected in the size of the tumour produced by the two specimens.

The final conclusion to be drawn from the experiments is, therefore, that the tumours arising from the inoculation of lipoid fractions of Rous sarcoma desiccates are to be attributed to contamination of the extracts by traces of the original tumour material.

SUMMARY.

The tumours produced by the lipoid material extracted from dried Rous No. 1 sarcoma are due to contamination of the lipoid with traces of the tumour desiccate. This lipoid material when freed from particulate matter has no carcinogenic properties.

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AN EFFECT OF GUINEA-PIG PASSAGE ON THE VIRUS OF RABIES.

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THE phenomenon described in this paper was first noticed some years ago during an attempt to discover by what route a strictly neurotropic virus, *e. g.* that of rabies, which presumably has little or no affinity for tissues other than nervous, infects the nervous system of rabbits after intravenous inoculation. At that time I was unaware of the work of Dold (1911) and Dold and Ogata (1912), showing that preparation of tissue suspensions with fresh, unfiltered serum prevents the sudden death so frequent when moderately large amounts of homologous tissues are inoculated intravenously. Hyde and Parsons (1926) had, however, recently restated the fact known to Dold that heterologous tissues were not nearly as " toxic " as homologous ; accordingly I passed to guinea-pigs the rabies virus which had previously been maintained by intracerebral passage in rabbits. It was at once apparent that the virus so obtained was vastly more potent by the intravenous route than was the rabbit virus, even though on intracerebral injection any difference between the two was often in favour of the latter. The difference between the two viruses was best expressed by determining the ratio between the minimal intracerebral (M.C.I.D.) and the minimal intravenous (M.I.I.D.) infecting doses.

Although Pasteur knew that passage of a street rabies virus through guinea-pigs enhances its virulence much more rapidly than does passage through rabbits, and it is a common practice with some workers to interpolate an occasional guinea-pig passage in maintaining a *virus fixe*, there seems to be recorded in the literature no observation comparable with the present.

The following pages detail the progress made in investigating the phenomenon before I left for Australia, where rabies virus is not allowed to enter.

TECHNIQUE.

In the experiments described below, 10 p.c. suspensions of fresh, unglycerinated brain were prepared with fresh, unfiltered homologous serum. After this had been centrifuged at 3000 to 4000 r.p.m. for 15 minutes, dilutions of

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the supernatant fluid were made in the usual manner with saline. When the suspensions have been prepared with serum, it is often, but not always, possible to inject as much as 10 c.c. intravenously in a rabbit without accident. Rabbits (1200 to 1500 g.) were inoculated intracerebrally or into the ear vein; in the latter event the ear was seared to destroy completely the needle track, into which traces of virus might have leaked. In guinea-pigs (350 g.) intracardiac injection, with precautions to prevent infection of the needle track (Hurst and Hurst, 1928), replaced intravenous injection. At each dilution near the minimal effective, two or three animals were used; the end-point was taken as the dilution at which one of two or two of three animals became infected, provided that all receiving the next lower dilution in the series succumbed. It is well known that, under these conditions, occasional animals receiving intracerebrally a very small amount of virus succumb when two or three receiving ten times the dose all survive; except with recently isolated strains of street virus (*e. g.* the Mor. virus), this occurrence is infrequent, and was disregarded in determining the minimal intracerebral dose. Sufficient observations were made to render it certain that these irregularities were in no way responsible for the results obtained. Where necessary, inoculated animals were observed over a period of six months.

Rabid animals intended for passage were not allowed to die. Rabbits were killed on the third day of symptoms; the titre of many of these brains was thus possibly not as high as might have been the case at death. Guinea-pigs were killed on the day on which they first showed definite symptoms, as with some of the viruses employed it was unusual for these animals to live 24 hours from the onset of the nervous disease. Some of the viruses had been preserved in glycerol for periods up to two years, and the early passages of these yielded brains of low titre.

The history of the viruses investigated is sketched below; I have to express indebtedness to Dr. J. Bailly for the Moroccan street virus.

T4: Trinidad virus of animal origin (Hurst and Pawan, 1932). Isolated in the monkey (*M. mulatta*) and thereafter passed through 16 rabbits, with a constant incubation period in the later passages. Preserved in glycerol for two years.

V.F.: *Virus fixe* (Paris strain) passed repeatedly in rabbits. Preserved in glycerol for two years.

Pr.: Isolated in 1933 in the guinea-pig from a case of paralytic canine rabies occurring at Princetown, N.J., and passed through three generations of guinea-pigs. Preserved in glycerol for nine months.

Mor.: Moroccan street (canine) virus in its first rabbit passage. Isolated in 1934 by Dr. J. Bailly.

EXPERIMENTAL FINDINGS.

Chart I figures the most extensive series of observations made with the Trinidad virus; rabbit and guinea-pig virus was passed to both species. At the first rabbit passage the titre of the virus was very low, and the intravenous injection of even 10 c.c. of a 10 p.c. suspension did not lead to infection; in

consequence the M.C.I.D. : M.I.I.D. ratio was not determinable. The brain of the first guinea-pig passage was of much higher titre. With continued passage the titre rose in both cases, but whereas the M.C.I.D. : M.I.I.D. ratio of rabbit brain after six passages was still in the region of 1 : 25,000 for rabbits and 1 : 6500 for guinea-pigs, that of guinea-pig brain was 1 : 50 to 1 : 500 for rabbits and 1 : 300 for guinea-pigs. A difference was already apparent after one guinea-pig passage, but curiously enough in this experiment was less at the second passage. The middle panels in the chart, illustrating the effect of alternate passages in guinea-pigs and rabbits, suggests that sometimes the effect produced by passage in the guinea-pig may persist after one rabbit

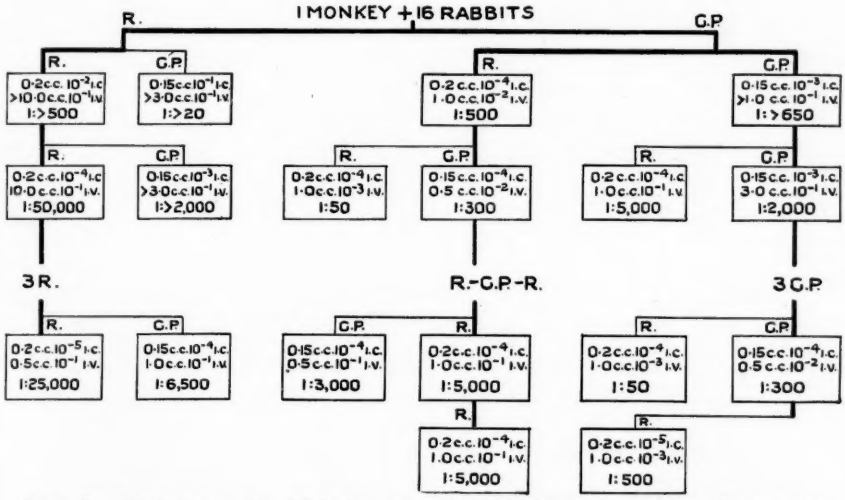

CHART I.—Trinidad virus, T4. Mininal cerebral and minimal intravenous infecting doses at each passage, with M.C.I.D. : M.I.I.D. ratios.

passage (third panel from the left, second row from the top), but more often does not.

That the effect of guinea-pig passage revealed above is far from being a chance occurrence is shown by further observations summarized in Table I. These are less comprehensive than the preceding, and are confined largely to the rabbit. The effect was seen again with the Trinidad virus, as also with the Princeton strain and the *virus fixe* (two separate series of tests). It was often, but not invariably, seen after a single guinea-pig passage ; equally low ratios were never obtained with rabbit brain. On the other hand, the effect was not apparent with the Moroccan virus when first isolated, though it was later, after a number of rabbit passages. As already mentioned, the results in the first series of tests with this virus were difficult to read owing to the irregularity of response of animals inoculated intracerebrally, but it seemed that at the

TABLE I.—*Behaviour of Various Strains of Rabies Virus after Passage through Rabbits and through Guinea-pigs.*

Strain.	Passages.	Infecting material.	Test animal.	Minimal intracerebral dose.	Minimal intravenous dose.	Ratio.
T4 .	1 M. + 16 R. + 1 G.P.	Guinea-pig brain	Rabbit	$0{\cdot}2$ c.c. 10^{-3}	$<1{\cdot}0$ c.c. 10^{-2*}	1 : <50
	1 M. + 16 R. + 2 G.P.	,,	,,	$0{\cdot}2$ c.c. 10^{-3}	$<1{\cdot}0$ c.c. 10^{-3*}	1 : <5
Pr. .	3 G.P. + 1 R + 2 G.P.	,,	,,	$0{\cdot}2$ c.c. 10^{-3}	$1{\cdot}0$ c.c. 10^{-1}	1 : 500
	3 G.P. + 1 R.	Rabbit brain	Guinea-pig	$0{\cdot}15$ c.c. 10^{-4}	$>2{\cdot}0$ c.c. 10^{-1*}	1 : >13,000
V.F. .	nR + 1 G.P.	Guinea-pig brain	Rabbit	$0{\cdot}2$ c.c. 10^{-4}	$>5{\cdot}0$ c.c. 10^{-1*}	1 : >25,000
	nR + 1 G.P.	,,	,,	$0{\cdot}2$ c.c. 10^{-5}	$<1{\cdot}0$ c.c. 10^{-2*}	1 : <5000
	nR. + 1 G.P. + 1 R.	Rabbit brain	Guinea-pig	$0{\cdot}15$ c.c. 10^{-4}	$>5{\cdot}0$ c.c. 10^{-1*}	1 : >33,000
	nR. + 1 G.P. + 1 R. + 1 G.P.	Guinea-pig brain	Rabbit	$0{\cdot}2$ c.c. 10^{-5}	$1{\cdot}0$ c.c. 10^{-3}	1 : 500
V.F. .	nR. + 1 R.	Rabbit brain	,,	$0{\cdot}2$ c.c. 10^{-5}	$1{\cdot}0$ c.c. 10^{-1}	1 : 50,000
	nR. + 1 G.P.	Guinea-pig brain	,,	$0{\cdot}2$ c.c. 10^{-5}	$1{\cdot}0$ c.c. 10^{-3}	1 : 500
	nR. + 2 R.	Rabbit brain	,,	$0{\cdot}2$ c.c. 10^{-4}	$>5{\cdot}0$ c.c. 10^{-1*}	1 : >25,000
	nR. + 2 G.P.	Guinea-pig brain	,,	$0{\cdot}2$ c.c. 10^{-5}	$1{\cdot}0$ c.c. 10^{-4}	1 : 50
	nR. + 2 R.	Rabbit brain and added normal guinea-pig brain	,,	$0{\cdot}2$ c.c. 10^{-4}	$1{\cdot}0$ c.c. 10^{-1}	1 : 5000
	nR. + 2 G.P.	Guinea-pig brain in glycerol 1 month	,,	$0{\cdot}2$ c.c. 10^{-4}	$1{\cdot}0$ c.c. 10^{-2}	1 : 500
	nR. + 2 G.P.	Guinea-pig brain in glycerol 5 months	,,	$0{\cdot}2$ c.c. 10^{-5}	$5{\cdot}0$ c.c. 10^{-1}	1 : 250,000
Mor. .	1 R. + 1 R.	Rabbit brain	,,	$0{\cdot}2$ c.c. 10^{-4}	$1{\cdot}0$ c.c. 10^{-2}	1 : 500
	1 R. + 1 R.	,,	Guinea-pig	$0{\cdot}15$ c.c. 10^{-4}	$0{\cdot}5$ c.c. 10^{-2}	1 : 300
	1 R. + 1 R. + 1 G.P.	Guinea-pig brain	Rabbit	$0{\cdot}2$ c.c. 10^{-5}	$1{\cdot}0$ c.c. 10^{-1}	1 : 50,000
	1 R. + 2 R.	Rabbit brain	,,	$0{\cdot}2$ c.c. 10^{-4}	$10{\cdot}0$ c.c. 10^{-1}	1 : 50,000
	1 R. + 1 R. + 3 G.P.	Guinea-pig brain	,,	$0{\cdot}2$ c.c. 10^{-4}	$5{\cdot}0$ c.c. 10^{-1}	1 : 25,000
	1 R. + 4 R.	Rabbit brain	,,	$0{\cdot}2$ c.c. 10^{-5}	$1{\cdot}0$ c.c. 10^{-2}	1 : 5000
Mor. .	1 R. + 9 R.	,,	,,	$0{\cdot}2$ c.c. 10^{-4}	$0{\cdot}5$ c.c. 10^{-1}	1 : 2500
	1 R. + 8 R. + 1 G.P.	Guinea-pig brain	,,	$0{\cdot}2$ c.c. 10^{-4}	$1{\cdot}0$ c.c. 10^{-3}	1 : 50
	1 R. + 11 R.	Rabbit brain	,,	$0{\cdot}2$ c.c. 10^{-4}	$5{\cdot}0$ c.c. 10^{-1}	1 : 25,000
	1 R. + 8 R. + 3 G.P.	Guinea-pig brain	,,	$0{\cdot}2$ c.c. 10^{-5}	$1{\cdot}0$ c.c. 10^{-3}	1 : 500

M. = monkey. R. = rabbit. G.P. = guinea-pig. 10^{-1} = a 10 p.c. suspension, and so on.
* indicates that the dose mentioned was the largest or smallest administered (as the case may be) in a particular test.
In the second column from the left the passages underlined were those made during the present investigation; those not underlined represent the previous history of the virus (where known).

second rabbit passage the virus behaved like guinea-pig virus, while at the passages immediately following this both rabbit and guinea-pig viruses behaved like rabbit virus.

With *virus fixe* the effect was noted in one test with virus which had been stored for one month in glycerol, as well as with fresh material, but not with the same material stored for five months in glycerol.

As stated previously, in most cases young rabbits were used for this investigation. In several instances, however, parallel observations were made with both young and adult animals. Although the results were not as regular in the mature groups, and the effect was less, more than 50 p.c. of these rabbits succumbed to intravenous doses of guinea-pig virus much below those necessary to produce infection with rabbit virus.

To exclude the possibility that the effect might be due to some property inherent in the guinea-pig brain itself, one sample of rabbit virus was emulsified in a centrifuged 10 p.c. suspension of normal guinea-pig brain in guinea-pig serum. The ratio in this experiment was 1 : 5000.

The possibility that the greater virulence of guinea-pig brain given intravenously was due to its possessing a larger number of infective particles per unit weight is ruled out by the titres for intracerebral inoculation of the various brains, as well as by the fact that to exclude this fallacy the results are expressed in the form of ratios.

In the further analysis of this phenomenon, two facts were ascertained by intramuscular inoculation into the calf of young rabbits of preparations of rabies virus in homologous serum exactly similar to those above. The suspensions were tinged slightly with finely divided vegetable charcoal, which permitted accurate removal, at various intervals, of the whole site of inoculation. From this, suspensions were prepared and inoculated intracerebrally into mice or guinea-pigs. Two or three rabbits were killed at each time mentioned in Table II, and the sites of inoculation pooled. From the weight of muscle removed, the titre of the suspension prepared from it, and the volume of the inoculum administered to the test animals, it could be ascertained how many infective units remained after a given period at the site of inoculation. The first fact established was that there was no evidence of local multiplication of either virus, making it improbable that the effect under consideration was due to the guinea-pig virus having acquired the power of multiplication in extraneural tissues. The second fact emerging was that the effect is seen also after intramuscular inoculation, since of the control rabbits which were not sacrificed a higher proportion developed rabies, and in a shorter average time, with guinea-pig than with rabbit virus; this can be seen by comparing columns 2, 3, 5 and 6, in all of which the initial doses were roughly the same. At this point it would have been very useful to have had figures for the average incubation period after intracerebral inoculation of the different viruses, but, on referring to the notes available, it appeared that in only a relatively small number was information sufficiently precise. The figures are given for what they are worth in Table III, where it is seen that only for the Trinidad virus was there a suggestion that the incubation period was less with guinea-pig than with rabbit virus; with *virus fixe* and with the Mor. virus no difference was apparent.

* indicates that the dose mentioned was the largest or smallest administered (as the case may be) in a particular test.
In the second column from the left the passages underlined were those made during the present investigation; those not underlined represent the previous history of the virus (where known).

TABLE II.—*Disappearance of Rabies Virus from an Intramuscular Site of Inoculation in the Rabbit.*

	1.	2.	3.	4.	5.	6.
Material inoculated	T4 : 7 G.P. passages.	V.F. : 3 G.P. passages.	V.F. : 3 R. passages.	V.F. : 4 G.P. passages.	V.F. : 2 G.P. + 2 R. + 1 G.P. passages.	V.F. : 2 G.P. + 3 R. passages.
Site of inoculation titrated in	Mice.	Mice.	Guinea-pigs.	Guinea-pigs.	Guinea-pigs.	Guinea-pigs.
Interval after inoculation :						
½ hour	10^{-3} (37,000)	10^{-2} (3000)	10^{-3} (10,000)	10^{-4} (63,000)	10^{-3} (7000)	10^{-3} (5850)
1 day	10^{-2} (6000)	0	10^{-1} (100)	10^{-2} (800)	10^{-1} (150)	10^{-2} (780)
2 days	0	0	10^{-1} (100)	10^{-1} (70)	10^{-1} (70)	0
3 „	10^{-1} (100)	0	0	10^{-1} (55)	10^{-1} (60)	tr.
5 „	10^{-1} (100)	0	0	0	tr.	0
7 „	10^{-1} (100)	0	0	0	0	0
9 „	tr.	.....	tr.	.....	.....	.....
11 „	0	0	.....	0	.....	.....
Controls	Two + 7 One + 8 One + 9 Two remained well	Three + 9 One + 10 One + 12 One remained well	One + 20 Four remained well	Five + 8 Two + 9 One + 10 One + 12	One + 9 Four + 10 One + 12 Three remained well	One + 11 One + 13 One + 14 One + 15 One + 16 One + 17 Nine remained well

Summary of controls.—24 of 31 receiving guinea-pig virus developed rabies in an average time of 9·2 days.
7 of 20 receiving rabbit virus developed rabies in an average time of 15·1 days.

The figures above show the titre of the suspensions prepared from the site of inoculation at various intervals. The figures in brackets show the calculated number of M.I.D. in these suspensions.

tr. = only one of two or three test animals succumbed. ..... = no test made on this day.

TABLE III.—*Average Periods in Days after Intracerebral Inoculation at which Rabic Symptoms were Definite in Rabbits.*

Virus.	M.I.D.	Guinea-pig brain. Number of observations.	Variation.	Average.	Rabbit brain. Number of observations.	Variation.	Average.
V.F.	10	9	7 to 10	8·8	1	.....	8·0
	100	8	7 „ 8	7·6	1	.....	7·0
T4	10	8	8 „ 12	9·6	6	10 to 11	10·3
	100	3	8 „ 9	8·6	4	9 „ 10	9·75
Mor.	10	3	10 „ 11	10·6	5	10 „ 14	11·0
	100	2	.....	11·0	4	9 „ 10	9·25

In 1935, Lennette and Hudson showed that poliomyelitis virus given intravenously to monkeys produced the nervous disease only if the central olfactory apparatus were intact. They suggested that the virus was excreted on to the nasal mucosa, and thence passed from the terminals of the olfactory nerves to the central nervous system ; as confirmatory evidence they found the virus in the nasal washings after intravenous inoculation. The virus of equine encephalomyelitis reaches the brain in the same manner (Hurst, 1936). It seemed, therefore, that a similar occurrence would explain the success of intravenous inoculation in the case of rabies virus, and the remaining experiments were performed with this in mind.

In view of the relatively low pathogenicity of *virus fixe* given by peripheral routes (as would have to be the case with unfiltered nasal washings), and the difficulty in filtering rabies virus, it seemed unprofitable to attempt to demonstrate excretion on to the nasal mucosa, and the experiment was limited to the intravenous inoculation of guinea-pig and rabbit virus into rabbits from which the olfactory bulbs had been removed three months previously. The results were unexpected, in that of animals receiving guinea-pig virus a higher proportion (16 to 20) of those surgically treated developed rabies than of controls of the same age and weight (12 to 20). This greater sensitivity of the former group was seen particularly well with the lowest dose of virus, 5 of 6 surgically treated animals succumbing as against 0 of 3 controls. Of 5 surgically treated and 5 control rabbits receiving rabbit virus intravenously, all survived. The noses of a number of the animals developing rabies were instilled with a mixture of sodium ferrocyanide and iron ammonium citrate ; half an hour later the rabbits were killed, and the head, with the cranial cavity opened, fixed in formalin containing hydrochloric acid. In all the control rabbits so treated, the olfactory bulbs were stained a definite blue ; in none of surgically treated animals was the scar-tissue at the site of operation coloured, showing that lymphatic connection with the nose had been interrupted, just as had the neural. It seemed, therefore, that the most likely explanation of the results was that in the latter group the hæmato-encephalic barrier at the site of trauma was still abnormal, and that infection of the nervous system had occurred at this point.

SUMMARY.

At the moment, therefore, the position is as follows : In both the rabbit and the guinea-pig rabies follows intravenous and intramuscular inoculation much more constantly and readily if the infecting material is guinea-pig brain than if it is rabbit brain. This phenomenon is not due to any property inherent in guinea-pig brain *per se*. The ratio between the minimal cerebral infecting dose and the minimal intravenous infecting dose is much lower with guinea-pig virus ; by expressing the observations in this form, it is at once made obvious that the number of infective units of guinea-pig virus needed to infect by the intravenous route is much smaller than of rabbit virus, and the former can be said truly to be more virulent for peripheral inoculation than the latter. It will be noted that this effect of guinea-pig passage was seen only with viruses which were some passages removed from street virus ; when recently isolated,

the Moroccan strain apparently behaved in a quite different manner, though, as explained, the results here were highly irregular and difficult to read. No evidence was obtained of local multiplication of guinea-pig (or rabbit) virus at a site of intramuscular inoculation.

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THE ENCAPSULATION OF HÆMOLYTIC STREPTOCOCCI.

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HISS (1905) described a method by which capsules could be demonstrated on all strains of *Streptococcus pyogenes*. He stated that in some cultures the capsules were quite as sharp and as well defined as those of the pneumococcus, while in others they were less well marked. Although these observations were supported by excellent photographs, they have never been completely accepted. Buerger (1907) described a method by which he could show capsules on at least 25 p.c. of the pyogenic streptococci isolated in his laboratory. He could furthermore predict the presence of capsules from the profuse, watery growth on glucose serum-agar. Numerous later investigators have commented on the occasional occurrence of encapsulated strains, but there has been little tendency to ascribe this property to hæmolytic streptococci in general. In 1934 Dawson and Olmstead reported on the mucoid phase of hæmolytic streptococci, and expressed the opinion that mucoid and capsulated organisms represent one of the chief variant forms of all hæmolytic streptococci. A similar conception was advanced independently by Pilot (1934), and Seastone (1934) also reported that large capsules could be demonstrated by Wright's stain on pyogenic streptococci grown in serum broth or whole rabbit's blood. By this method capsules could be demonstrated only during the first 2 or $2\frac{1}{2}$

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hours of growth. Nevertheless, many investigators have either been unable to confirm these observations, or have been unable to obtain consistent results by the methods described. Much of the confusion appears to be due to the failure to recognize the existence of distinct serological groups (Lancefield, 1933) within the hæmolytic streptococci. The whole subject has therefore been critically re-examined, and the following procedure is offered as one which gives uniformly reliable and consistent results.

TECHNIQUE.

Preparation of cultures.—Neopeptone beef infusion agar containing 0·2 p.c. dextrose and 2·5 p.c. rabbit's blood is used. Freshly poured plates are essential in order to ensure an adequate amount of moisture. The dextrose is added to the agar at the same time as the blood. The organisms are grown on this medium either for 3 to 5 hours at 37° C., or for 10 to 12 hours at room temperature.

Preparation of reagents.—A 4·8 p.c. solution of alcoholic (C_2H_5OH, 95 p.c.) gentian violet is kept as the stock solution. For use, 5 c.c. of this solution is added to 95 c.c. of distilled water, and the final solution then passed through filter-paper.

Method of staining.—(*a*) Make a thin smear by emulsifying the organisms in a loopful of serum ; allow to stand for 10 to 15 seconds and then spread over the slide. (*b*) Dry in air and fix gently with heat. The slide should never be heated enough to warm it appreciably. (*c*) Flood with gentian violet and heat until the dye just begins to steam—no longer. (*d*) Wash quickly with a small amount of 20 p.c. copper sulphate and dry by blotting. Care should be taken that the slide is completely dry within 5 seconds after the copper sulphate is first applied.

The foregoing procedure is essentially the same as the well-known capsule stain described by Hiss in 1905. In employing this technique, however, it is important to use as little heat and as little copper sulphate as possible. The capsules on hæmolytic streptococci are more readily destroyed by both these factors than are those on pneumococci.

RESULTS.

By this procedure distinct and well-defined capsules have been demonstrated on the mucoid phase of hæmolytic streptococci belonging to Groups A to F inclusive. Capsules occasionally have been demonstrated on mucoid strains of Groups B and C even after 15 or 18 hours' incubation at 37° C. Dawson, Hobby and Olmstead (1936) have previously shown that the mucoid phase constitutes one of the chief variant phases of the various groups of this organism. Capsules have not been demonstrated on strains belonging to Group G, probably owing to the fact that the mucoid phase in this group has not yet been identified. Capsules can be demonstrated most readily in smears made from young mucoid colonies, but they can also be seen frequently in the heart's blood of infected mice.

The appearance of the capsules on strains belonging to Groups A, B and C is shown in Figs. 1, 2 and 3. The capsules on Group A strains are large, and at times sharp and well defined. At other times they appear to be "in a semi-fluid state and on the point of dissolving" (Hiss, 1905). When several organisms are massed together, their capsules seem to coalesce. The capsules of Group C strains are very similar to those of Group A, but as a rule they are more clearly defined. In Groups B and D the capsules are smaller, more sharply defined and closely resemble those of pneumoccoci. In Groups E and F the capsules are very small but distinct.

Encapsulation bears the same relation to virulence and other properties of the hæmolytic streptococci as does the mucoid phase. This is discussed in detail elsewhere (Dawson, Hobby and Olmstead, 1937). It is interesting to record here, however, that capsules can be demonstrated on all strains of hæmolytic streptococci which possess a high degree of virulence for white mice, irrespective of the group to which they belong. Furthermore, the majority of Group A strains isolated from pathogenic conditions in the human are mucoid and encapsulated on primary culture. Only occasional strains have been encountered which could not be transformed to the mucoid phase when transferred on a suitable medium.

SUMMARY.

1. A method is described by which capsules can be demonstrated on hæmolytic streptococci, Groups A to F, while the organisms are in the mucoid phase.

2. Group A hæmolytic streptococci when isolated from disease conditions in the human are usually encapsulated and in the mucoid phase.

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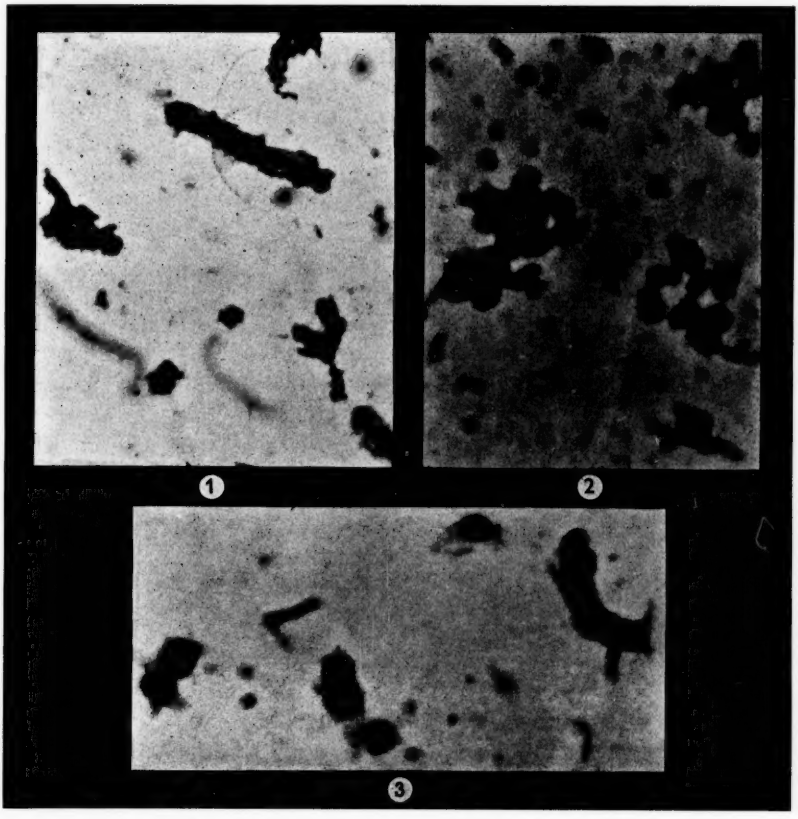

Streptococcus hæmolyticus. Mucoid organisms showing encapsulation. × 5040. Fig. 1, Group A, strain C203 MV. Fig. 2, Group B, strain 090. Fig. 3, Group C, strain K64.

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THE EFFECTS OF ROAD DUST " FREED " FROM TAR PRODUCTS UPON THE INCIDENCE OF PRIMARY LUNG-TUMOURS OF MICE.

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In a previous paper (Campbell, 1934) it was shown that road dust containing 2–3 p.c. of tar causes cancer of the skin in 70 p.c. of mice surviving long enough, and increases the incidence of primary adenoma of the lung to tenfold that of the controls. The mice were exposed to the dust in a large respiratory chamber, the air of which was kept well mixed by means of a fan, driven by a motor outside the chamber. A cloud of dust, such as might arise from a dusty road, was produced in the chamber, about four to six times a day at hourly intervals during five days a week for about a year, that is, from the third to fifteenth month of the animal's life. The mice were thus exposed to a dust cloud on something less than 1500 occasions. The mice were allowed to live their allotted span of life, and then were examined carefully for any pathological changes, particularly as regards tumours of the lung. The lungs of the dusted mice contained much dust, more or less arranged in isolated clumps of phagocytes. The human lung often contains much dust.

In order to determine the part played by the tar as distinct from that due to the dust particles, the experiment has been repeated with a portion of the same dust from which the tar products were removed in the Soxhlet apparatus by means of benzene.

METHODS.

In general, the technique described for experiments in the previous paper (1934) was followed as closely as possible in order to make the two researches comparable, except as regards presence of tar products.

The removal of the tar from the dust by benzene required, with frequent stirring, 10 to 14 days for each sample (70 c.c.) in the Soxhlet larger apparatus, because the tar is very strongly adsorbed upon the dust particles. The extraction was continued until the extracting fluid became colourless and showed absence of tar after evaporation. Prof. J. W. Cook writes to us as follows: " Perhaps a more sensitive test is that, in the light of a screened mercury lamp, a benzene solution of benzpyrene—the carcinogenic constituent of tar—shows an intense violet fluorescence." This test was applied by us, and no fluorescence occurred, so that all of the benzpyrene must have been removed by prolonged extraction with benzene. It is of course possible, but not probable, that some carcinogenic agent still remained adsorbed to the dust.

Our first attempt to extract all the tar was not completely successful, and we used this partially tar-free dust with one batch of 79 mice for about a week,

but then stopped the dusting as, on closer examination, a small but definite amount of tar was detected in the dust. This group of mice thus received only one week's dusting with an almost tar-free dust; we have called this group of mice " Control B ". The true controls, called " Control A ", received no special dusting at all, but of course came into contact with dust from their food, and bedding (sawdust), and also with any dust in the atmosphere. After apparently complete extraction of the tar from the dust with benzene, another group of mice was dusted for about a year with this tar-free dust. This group of mice we have termed " Experimental " (Table I).

The dust was analysed as in the previous paper, and its composition was almost identical with that given for previous Experiment 2 (1934), with the exception that it now contained no obvious tar.

The routine for dusting the mice was the same as in the former research, so that the dust was not only breathed by the mice, but came into contact also with their hair and skin.

RESULTS.

As was expected, the tar-free dust did not give rise to warts or cancer of the skin, so that the tar-product which was the cause of the skin cancer in the previous experiment (1934) had apparently been removed by the benzene extraction. In the experiment with tar-free dust the skin appeared to keep healthy in spite of the prolonged dusting, and the dusted mice also grew at a fairly normal rate compared with that of controls in general. The controls in this experiment (Table I) showed rather better growth than some other controls have done (Campbell, 1936). The death-rate for the dusted mice was lower than that for the controls, until near the end of the experiment, when the lung-tumours were exerting their effect. The male mice usually showed a higher death-rate than the females, which were separated from them. Conclusions from differences in rate of growth and death-rate of mice must be drawn with care. The mice were kept in groups of 9 or 10, and the male mice may have fought amongst themselves, the weaker thus dying sooner. Starting with about 75 mice, 3 months old, there were usually about 55 alive at the age of 10 months. In Tables I and II the number of males is given as numerator and the number of females as denominator after each total.

As regards incidence of primary tumours of the lung, the mice exposed to tar-free dust showed a definite increase compared with the controls A (Table I). Also these tumours appeared somewhat earlier amongst the dead experimental mice than amongst the control A. " Control " B mice, which had only one week's dusting, with dust containing a very small amount of tar, showed a slight increase in incidence of lung-tumours compared with control A mice, which were not exposed at all to special dusting.

Table II records the details for the previous experiment (1934) with the road dust containing tar. Comparison of these results with those for the tar-free dust (Table I) reveals the fact that the tar not only further increases the incidence of the primary lung-tumours, but also accelerates their appearance. At the 500th day of the experiment with tar-free dust only 15 p.c., or 4 out of the 27 mice which had died since the 200th day of the experiment, possessed

TABLE I.—*Experimental Mice Exposed to Tar-free Dust.*

Date.	Number of days of experiment.	Number of mice alive.			Weight (g.) of living mice.			Animals with lung-tumours.		
		Control A.	"Control" B.	Experimental.	Control A.	"Control" B.	Experimental.	Control A.	"Control" B.	Experimental.
20.xi.34	0	77 (41/36)*	79 (36/43)	78 (41/37)	18·0	19·7	18·3	0	0	0
28.ii.35	100	61 (27/34)	71 (31/40)	73 (36/37)	22·7	22·0	22·0	0	0	0
8.vi.35	200	50 (19/31)	47 (15/32)	58 (29/29)	23·7	23·3	23·1	0	0	0
16.ix.35	300	37 (13/24)	38 (13/25)	49 (21/28)	27·8	25·5	25·5	0	0	0
25.xii.35	400	30 (11/19)	30 (10/20)	39 (17/22)	30·4	26·6	27·7	0	0	1
3.iv.36	500	25 (8/17)	22 (8/14)	31 (13/18)	32·6	27·8	27·7	0	0	4
11.vii.36	600	17 (5/12)	18 (7/11)	21 (9/12)	31·4	27·8	27·8	1	3	6
19.x.36	700	10 (1/9)	10 (5/5)	7 (2/5)	29·1	25·7	29·4	5	9	19
27.i.37	800	5 (1/4)	2 (1/1)	1 (0/1)	34·4	22·5	31·0	5	12	25
18.ii.37	822	5 (K) (1/4)	0	1 (K) (0/1)	33·6	..	29·0	10	14	26

* Males/females. K = Killed.

TABLE II.—*Experimental Mice Exposed to Tar Dust.*

Date.	Number of days of experiment.	Number of mice alive.		Weight (g.) of living mice.		Animals with lung tumours.	
		Control.	Experimental.	Control.	Experimental.	Control.	Experimental.
17.xi.32	0	69 (41/28)*	73 (27/46)	18·4	18·2	0	0
25.ii.33	100	57 (35/22)	63 (25/38)	24·9	24·4	0	0
4.vi.33	200	50 (29/21)	61 (24/37)	25·2	25·5	0	0
13.ix.33	300	40 (23/17)	60 (23/37)	27·5	27·0	0	0
22.xii.33	400	29 (17/12)	34 (11/23)	25·0	26·4	0	11
31.iii.34	500	23 (15/8)	21 (8/13)	26·0	23·2	0	24
13.vi.34	574	10 (10/0) (K)	2 (1/1) (K)	28·0	26·0	4	45

* Males/females. K = Killed.

lung-tumours, whereas with the mice exposed to the tar dust (Table II), 60 p.c., or 24 out of the 40 mice which died during the same period, 200th–500th day, revealed such tumours. We have taken the 200th day of the experiment in this estimation because at this time the mice were about 10 months old, being about 3 months old at the start of the experiment. Lung-tumours are very rare indeed in mice younger than 12 months ; in fact, they are usually not numerous until towards the end of the second year of life—that is, during the last quarter of the mouse's existence. This resembles the condition in man, where the mean age at death with lung-tumours is 55 years. In man lung-tumours are more prevalent in males than in females, perhaps because the former are more exposed to the agent. With the mice, the tumours were equally numerous among the two sexes, probably because both were equally exposed to the carcinogenic agent or " irritant " in the dust.

That our experimental mice were not particularly susceptible by heredity to tumour growth is evident from the incidence of mammary cancer (Table III). In all three batches of mice, totalling 234 (females 118), only 3 developed mammary cancer, and only 1 of these was accompanied by a metastasis, which was situated in the mediastinal tissue ; this mouse also possessed a primary lung tumour. None of the mice dusted for a year developed a mammary cancer.

TABLE III.—*Absolute Number of Mice with Pathological Changes.*

	Control A.	" Control " B.	Experiment.
Lung-tumours	10 (4/6)*	14 (7/7)	26 (11/15)
Metastasis	0	0	3 (3/0)
Mammary tumours	2 (0/2)	1 (0/1)	0
Metastasis	0	1	0
Lymphatic tissue increase	8 (5/3)	3 (2/1)	7 (4/3)
Mediastinal tissue increase	3 (2/1)	4 (1/3)	6 (2/4)
Broncho-pneumonia	3 (2/1)	6 (1/5)	13 (11/2)
Abscess (included in above)	1 (1/0)	4 (1/3)	5 (4/1)
Liver-cysts	24 (9/15)	27 (7/20)	23 (11/12)
Liver atrophy and necrosis	5 (0/5)	3 (3/0)	8 (4/4)

* Males/females.

Mice, well cared for, do not always appear to be greatly affected by the presence of a tumour. This holds not only for lung-tumours, but also for mammary cancer and for skin cancer produced by tar. Nevertheless, the influence of the higher incidence of lung-tumours amongst the dusted mice upon their death-rate is indicated from the 400th day onwards (Tables I and II).

We have also examined carefully the lymphatic tissue in the lung itself and in the mediastinum (Table III). There was no increase in lymphatic tissue in the lungs of the dusted mice compared with the controls, but there was some increase in the number of mice with hypertrophy of the mediastinal lymphatic tissue. Of course, the lymph-nodes near the trachea and bronchi

of the dusted mice contain much more dust than those in the controls ; but there is—apart from the tumours—no significant inflammatory reaction to the dust, either in the lung or in the lymph-glands. Nevertheless, there is an increase due to dust in the number of mice with broncho-pneumonia (Table III), and abscess formation followed in some of these. This increase in broncho-pneumonia was observed also in the previous experiment with tar dust. Terminal pneumonia and congestive changes are not included in the above ; they were more or less equally distributed amongst controls and experimental mice. The liver also showed considerable pathological changes, *e. g.* parasitic cysts, atrophy and necrosis, in all groups of mice without any special difference that could be attributed to the dust.

GENERAL ARRANGEMENT AND HISTOLOGY OF THE TUMOURS.

Although some of the numerous lung-tumours often seen in one mouse may be extensions from a primary lung-nodule, the general opinion, with which we agree, is that these lung-tumours are usually multiple in origin. This supports the view that the factor which originates the tumours comes from the external environment. In our mice dusted with road dust minus tar the number (26) of mice with lung-tumours is more than double that (10) in the controls, and also the number (7) of lung-tumours per dusted mouse with tumour is more than double that (3) in the controls. We consider that the tumours in the lungs of the controls are also due to a similar factor from the external environment.

In our specially dusted mice the tumours are also much larger than in the controls. The surface area (length by breadth as seen on the visceral pleura) of the largest lung-tumour in the dusted mice is 140 sq. mm., while that in the controls is only 36 sq. mm. ; the average figures for the largest tumour in each mouse with a tumour are 35 sq. mm. for the dusted mice and only 9 sq. mm. for the controls. The most important difference, however, is that there are far more with malignant tumours in the dusted mice than in the controls. Thus taking the number of mice (*a*) with malignant tumours, (*b*) with tumours showing commencing malignancy, and (*c*) with simple papillary adenomata, the figures for the dusted group are 14, 7 and 5 respectively, while for a similar total of control mice the figures are 2, 5 and 3 respectively. Further, among the dusted mice we discovered three with tumours giving rise to metastases—the first to be recorded in this country, we believe. The tumours are regarded as malignant if they give rise to metastases and also if, without metastases, they contain infiltrating spindle cancer-cells or polygonal or spheroidal cancer-cells, or if the cells are arranged as in the typical adeno-carcinoma. The tumours are regarded as showing commencing malignancy if their edges reveal definite evidence of change to atypical or cancerous cells. The simple tumours reveal hyperplasia with cells arranged as in the usual papillary adenomata. None of the tumours in the controls possessed spindle cancer-cells, whereas at least 7 of the dusted mice revealed tumours with such cells, and in 6 others there was evidence of change to this type of cell. In a malignant tumour all three stages are observed, *i. e.* some parts are non-malignant, others show commencing malignancy, and still others are malignant. The tumours appear

to originate usually from the alveolar cells as non-malignant tumours and then change more or less rapidly into the malignant type, depending on the degree of irritation. One tumour in one of our dusted mice appears to originate from the basal layer of a large bronchial tube, and to give rise almost at once to spindle-cells spreading along the bronchus into the lung-tissue and into the mediastinum, with a metastasis in the right kidney.

There is a considerable literature on the histology of mouse-lung tumours, which has been mentioned in our previous papers (Campbell, 1934, 1936). Many of our observations regarding the arrangement and histology agree with this earlier information. The only other research we have found which describes metastases is that of Maud Slye and her colleagues (1914) in America. They describe metastases in 4 mice out of a total of 160 with lung-tumours found in 4000 autopsies of mice of cancer age. Our own total to date is 3 mice with metastases out of 192 mice with lung-tumours observed in about 1000 mice —controls and experimental. Of the 4 mice with metastases described by the American workers 2 possessed metastases in the mediastinum, while in the other 2 there were also extensions or metastases in the chest-wall or in the diaphragm, with, in one case, a nodule in the kidney substance. These metastases, we understand, were in normal mice, *i. e.* mice not exposed to special experiment ; but Slye and her colleagues observed that their mice with lung-tumours often suffered also from an eye affection. They were not able to give any explanation for the eye condition, but in view of our finding that dust or some "irritant" in dust definitely increases the incidence of lung-tumours in mice, we suggest that both the eye condition and the lung-tumours in the American mice were due to some "irritant" either in the atmosphere or in the contents of the boxes containing the mice. We (1934) observed a similar eye condition in our mice exposed to dust from tarred roads, but not in the mice exposed to the same dust minus tar.

We give some photographs (by Mr. F. V. Welch, of Mr. J. E. Barnard's department) of portions of a primary tumour and the three metastases, the magnification being about 185 in all cases. Fig. 1 shows an area in a primary lung-tumour where transition from the ordinary adeno-carcinoma arrangement to the spindle cancer-cell is taking place ; the black areas are phagocytes with dust. In Fig. 2 the metastasis, consisting of similar spindle-cells, is seen infiltrating the heart-muscle substance near the front aspect between the right and left ventricles. We believe this is the first metastasis from a mouse lung-tumour to be observed in this organ. There was no metastasis in this case in the mediastinal tissue, that in the heart-substance being quite isolated.

Fig. 3 shows a portion of a metastasis in the cortex of the right kidney of the second mouse ; the resemblance to the primary tumours of the papillary adenomatous type is striking. Slye and her co-workers (1914) describe similar metastases in the mediastinal tissue and chest-wall. When there are nodules in these situations care must be taken to exclude the possibility of direct extension along the bronchi or vessels from the primary tumour.

In our third mouse with metastasis there are many columnar cells and also spindle-cells in the primary lung-tumour extending into the lung-substance and also along a bronchus into the mediastinum. In the right kidney cortex and in some of its blood-vessels (Fig. 4—see small artery) there are metastases

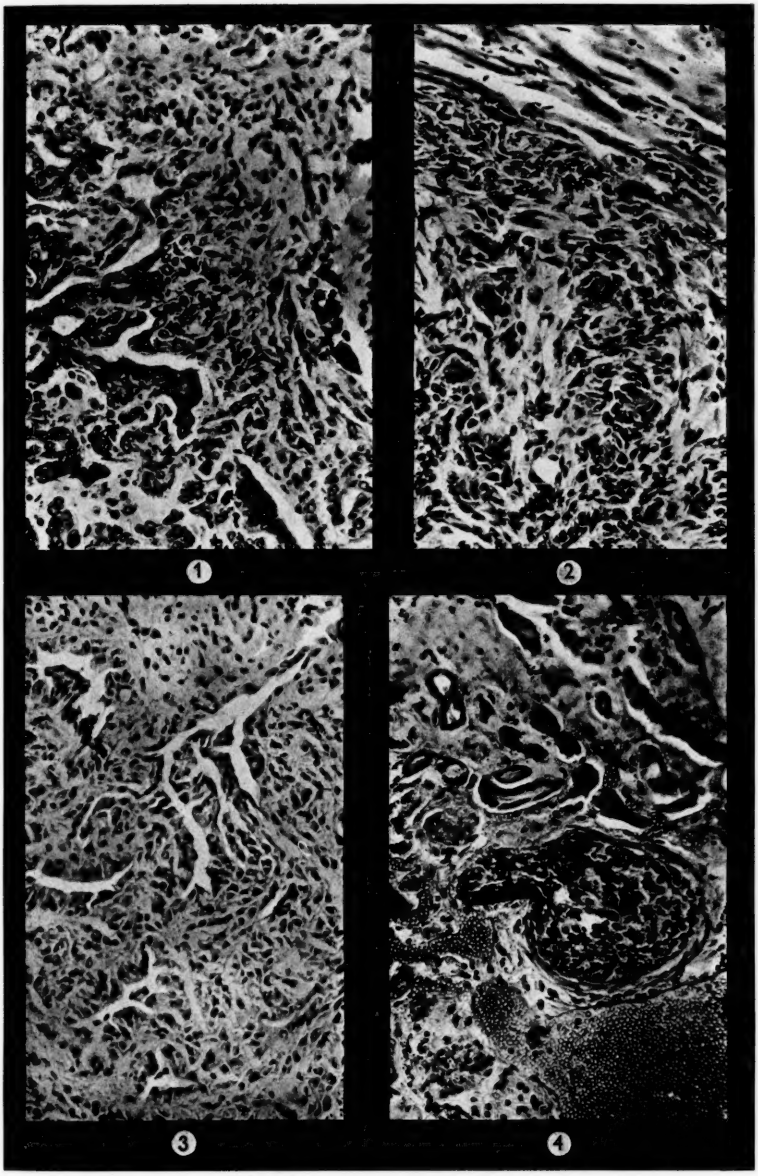

FIG. 1.—Primary adeno-carcinoma of lung, showing transition to spindle-cells. Black areas, collections of phagocytes with dust. H. and E. × 185.

FIG. 2.—Metastasis of spindle-cells in heart-muscle, from tumour in Fig. 1. H. and E. × 185.

FIG. 3.—Metastasis, from lung-tumour, in kidney cortex, showing papillomatous character. H. and E. × 185.

FIG. 4.—Metastasis of spindle-cells, from lung-tumour, in small artery of kidney, extending into larger artery. H. and E. × 185.

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of similar spindle-cells. Slye and her colleagues describe a similar metastasis in the kidney substance.

The sections illustrated were stained with hæmatoxylin and eosin. Sections were also stained with Weigert and Van Gieson's stains to demonstrate the arrangement of the elastic tissue. In the simple adenomata of the lung the elastic tissue, as Haaland (1911) found, is arranged in regular and rather thickened circles surrounding the alveoli. As the tumours grow and become malignant the elastic tissue becomes thinned and irregular in arrangement, and is often absent from the centre of the malignant tumour. Elastic tissue was not found in any of the metastases.

DISCUSSION.

In Table IV we have grouped together all the experiments we have completed dealing with the incidence of lung-tumours. This table contains only the incidence in mice living 10 months or longer. Each batch of mice consists of about 50–60 mice, all of the same mixed strain to exclude effects of heredity. In normal control mice the lung-tumour incidence increases with age and the incidence in control mice 10 months or older averages about 14 p.c. in a total of about 320 mice. The highest incidence for any batch of 55 control mice is 20 p.c.

TABLE IV.—*Results for Primary Lung-tumours of Mice Living* 10 *Months or Longer.*

Condition of mice.	Percentage of mice with lung-tumours.	Greatest age of mice (days).
Normal controls (A)	8	664
,, ,, (B)	8	705
,, ,, (C)	13	841
,, ,, (D)	14	849
,, ,, (E)	20	919
,, ,, (F)	20	912
Motor exhaust gas (1)	18	849
,, ,, ,, (2)	26	841
Mice dusted one week only	30	912
Cigarette smoke	30	919
Road dust minus tar	45	912
,, + tar + CO	47	705
,, + tar	74	664

Exhaust gases (Campbell, 1936) from internal combustion engines, in concentrations somewhat resembling those obtaining in traffic blocks and garages, do not influence to any extent the incidence (18–26 p.c.) of primary lung-tumours. Carbon monoxide itself has a retarding influence upon tumour growth (Campbell, 1932, 1933).

The incidence (30 p.c.) for the group of mice exposed to cigarette smoke (Campbell, 1936) containing some tarry matter is on the high side, but some of these mice survived longer than those in some others groups. Mice

(" Control " B present research) specially dusted for one week only, with dust containing a small amount of tar, also exhibit an incidence of 30 p.c.

Road dust minus tar (present research) definitely increases the incidence (45 p.c.) of lung-tumours, but not to so great an extent (74 p.c.) as when the tar is also present (Campbell, 1934). The composition of the inorganic part of the dust which formed 90 p.c. of the total weight is as follows : SiO_2, 44 p.c. ; CaO, 14 p.c. ; Fe_2O_3, 4.5 p.c. ; MgO, 2·5 p.c. ; Al_2O_3, 12 p.c. The silicon was present apparently as silicate, since it did not cause silicosis in the lungs of guinea-pigs. We have given the composition of the inorganic constituents, but these may—we shall test this later—have had little to do with the increase in incidence of lung-tumours. There may still have been some carcinogenic agent present after removal of the tar products by benzene. The presence of lung-tumours amongst some of the controls may be due to a similar carcinogenic agent or " irritant " present in the dust of the general atmosphere—perhaps from road tar-dust—or from dust in the bedding and food in the boxes. It may be a matter of chance as to any particular control mouse breathing in dust with a carcinogenic agent or " irritant ", or all the mice may not be equally susceptible to the agent. This factor of susceptibility may render difficult the drawing of conclusions from incidence in trades, factory workers, etc. Bittner (1935), Andervont (1937) and others have demonstrated the increase in susceptibility due to heredity when certain inbred strains of mice are employed. Even here we believe the stimulus to start the tumour comes from the external environment.

The highest incidence (74 p.c.) of lung-tumours in the mice is found (Table IV) in the group exposed to road dust containing tar (2–3 p.c.). In the other experiment with tar road-dust the tar content was 1·6 p.c., and carbon monoxide was breathed in low concentration at the same time ; the incidence for lung-tumours is 47 p.c. There is no doubt from these results and from other observers' work that tar, either by a direct action on the lung itself or indirectly by some effect on other tissues or organs, increases the incidence of lung-tumours in mice (for literature, see Campbell, 1934 and 1936).

Haaland (1911) regards parasites, *e. g.* nematodes, as a possible cause of lung tumours in mice ; but we, like Slye and her colleagues (1914), have found no evidence that such parasites have any particular relationship to the tumours.

There is evidence that lung-cancer is increasing in man, according to the ' Annual Report of the British Empire Cancer Campaign ', 1936, p. xx. This states than an abnormal liability to cancer of the lung appears to affect workers exposed to coal, gas and tar, and those engaged in the preparation and sale of tobacco. Occupations where there is a special exposure to road dust also show a somewhat high incidence of cancer of the lung (Kennaway, 1936). In general, the above experimental results with mice confirm these findings for man.

It has been suggested (Kennaway, 1936) that, as there is no evidence that chimney-soot tar increases lung-tumours in man, the tar in dust from roads is not likely to do so. This argument will only hold if the tar from chimney soot contains as potent a carcinogenic agent as the tar in road-dust. We are testing this now, by painting mice with the extracted tar, and also by carrying out experiments in which mice are dusted with chimney soot, and also with

soot from internal combustion engines burning heavy oils. It must be emphasized that such experiments are of little value unless the mice live nearly two years, since lung-tumours do not occur in any number during the first year of life. As is the case in man, the time factor is important.

It is well known that man, like the mouse, is sensitive to tar as regards skin-cancer. We have shown above that tar increases the incidence of lung-tumours in mice; tar is, therefore, likely to influence the incidence of human lung-tumours. It was formerly held by some that, as mouse lung-tumours did not give rise to metastases, they did not resemble human lung-tumours. It has been shown above that mouse lung-tumours may give rise to metastases, and others (*e. g.* Lastra and co-workers, 1935) have recorded human lung-tumours without metastases, therefore there is no difference between man and mouse in respect to metastases.

SUMMARY.

(1) It was shown previously that dust from tarred roads causes cancer of the skin of mice. Road dust minus tar, as removed by benzene, does not cause cancer of the skin of mice; it stimulates the production of lung-tumours in mice, but not to so great an extent as when the tar is also present. Lung-tumours are rare in mice before they are 12 months of age.

(2) The dust does not cause any obvious inflammatory reaction—apart from the tumours themselves—in the lung or lymph-glands.

(3) Perhaps the presence of lung-tumours amongst some of the control mice is due in part to the dust breathed from the contents of their boxes or from the general atmosphere. This dust may contain a carcinogenic agent, *e. g.* tar, or it may "irritate" the lung without obvious inflammatory reaction.

(4) Tar, either by a direct action on the lung itself or indirectly by some action on other tissues, or both, stimulates the production of lung-tumours in mice and hastens their appearance.

(5) Metastases from lung-tumours in mice are recorded, for the first time in this country, so far as we know. The majority of the lung-tumours in the dusted mice are malignant.

I am indebted to Sir Patrick Laidlaw, Dr. J. A. Murray and Dr. J. W. Purdy for examination of some of the tumours and the metastases. Thanks are due also to my assistant, Mr. C. Pergande, for much help throughout this research.

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A SEROLOGICAL ANALYSIS OF THE PROTECTIVE SUBSTANCES IN SPECIFIC ANTIBACTERIAL SERA WHICH CONTROL EXPERIMENTAL INFECTION WITH *CL. ŒDEMATIS MALIGNI* (*VIBRION SEPTIQUE*).

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THE fact that a specific antibacterial immunity can be established against experimental infection with *Vibrion septique* has been demonstrated by Robertson and Felix (1930) and Henderson (1934, 1935). The immunizing agent used by these workers consisted of the heat-stable component of the bacterial cell, that is the O antigen, but more recent experiments (Henderson, 1935) have indicated that labile cell components may also play a part in stimulating the production of protective substances. The results of passive protection tests in mice have shown that a rabbit serum prepared by immunizing with the O antigen of *Vibrion septique* may have a consistently lower protective value than a serum prepared by immunizing with a formolized unheated culture. This type of serum contains antitoxin in addition to antibacterial substances, but the results obtained suggest that neither antitoxin nor O antibody is responsible for the additional protective value. For example, an amount of antitoxin at least five times greater than that present in the combined antibacterial-antitoxic serum fails to protect mice against the test dose of "activated" spore-suspension. Again, after complete removal, by absorption, of the O antibody a strong protective element remains. The experiments described in the following pages are concerned with a further investigation of the nature of the antigen which is responsible for stimulating the production of antibacterial substances other than O antibody.

Felix and Robertson (1928) and Henderson (1934) have shown that *Vibrion septique* possesses a heat-stable O antigen and a heat-labile H antigen. The O antigen withstands heating for at least 2½ hours at 100° C. The heat lability of the H antigen has not been accurately determined, but it is found to be gradually destroyed, when examined either as a hapten or as a complete antigen at temperatures above 60°–70° C. In the preparation of H antigen the usual practice has been to heat the suspension for 1 hour at 60° C.; this procedure destroys any toxin present and kills the vegetative forms of the organism but leaves the spores undamaged; in this state the antigen is comparatively innocuous for rabbits and the H antigen retains its agglutinogenic

activity. Temperatures as low as 60° C. may, however, induce qualitative changes in the heat-labile cell components without causing actual destruction. For this reason, in the experiments to be described, the heating of such suspensions was avoided, and they were rendered innocuous to rabbits by the addition of small amounts of formaldehyde. Throughout the text of the paper this type of antigen and the corresponding serum from rabbits immunized with it are designated by the letter F. The distinction has been made necessary in order to avoid confusion with H antigen and antibody. As suggested above, the unheated antigen may include labile cell components different from that which is recognized by in-vitro tests as H antigen.

MATERIALS AND METHODS.

The strains of *Vibrion septique* employed have been described previously (Henderson, 1934).

Methods for (1) the preparation of the O antigen, (2) the preparation of a spore-suspension for experimental infection, (3) the immunization of rabbits with O antigen, (4) passive protection tests in mice, (5) in-vitro tests and (6) absorption experiments were similar to those used in the earlier work (Henderson, 1934, 1935). The antitoxin content of the sera was estimated by the mouse intravenous method and the values are expressed in international units.

Rabbits of the Agouti variety weighing 3–4 kg. were selected for the preparation of serum, and mice of mixed breed weighing 18–22 g. were used for passive protective experiments.

Modifications of technique and the preparation of special antigens are described in the text.

The preparation of formolized (F) suspension.—The organisms were grown under atmospheric conditions at 37° C. for 18–24 hours in a tryptic digest broth (pH 7·6) containing 1 p.c. glucose and a small amount of minced meat. Growth deposits, obtained by centrifuging, were washed twice in a large volume of distilled water, and finally resuspended to opacity 5 (Brown's scale) in 0·85 p.c. NaCl solution to which 0·2 p.c. of 40 p.c. formaldehyde was added. The suspension was stored for 48 hours at room temperature before use, and thereafter at $\pm$ 1° C. for a period of not more than 6 days.

The preparation of F serum in rabbits.—A sample of normal serum was collected from all animals by bleeding from the jugular vein. The F antigen was given intravenously in four doses (0·5 c.c., 1·0 c.c., 2·0 c.c., 2·0 c.c.) separated by intervals of 4–5 days. A bleeding was taken 6–7 days after the last injection of vaccine. When necessary the course of immunization was repeated.

The in-vitro differentiation of F and O sera.—Agglutination and agglutinin-absorption tests alone were used. The agglutinins detectable in an F serum after complete absorption with O antigen have been taken as the in-vitro index of the F antibody content. F suspension in the presence of O-absorbed F serum gave agglutination typical of the H type described by Felix and Robertson (1928). All attempts to distinguish by in-vitro methods two or more

antibodies associated with labile cell components failed. The differentiation of F and H antigens and antibodies, therefore, is thus far an artificial one, and has been adopted only as a matter of convenience in description.

A COMPARISON OF THE PROTECTIVE VALUE OF F AND O SERA FOR MICE.

F and O antigens from the classical strain "Pasteur" were used in the production of the two types of serum. Groups of mice were injected intravenously with graduated amounts of each serum. Twenty-four hours later they were given a test dose of an "activated" homologous spore-suspension by the intradermal route (Henderson, 1935). A group of mice each receiving a dose of normal rabbit serum and a group of mice each receiving 10 units of "pure" antitoxin from the horse were included as controls. The results are summarized in Table I.

TABLE I.—*The Relative Protective Value of F and O Sera.*

Type.	Serum. O agglutinin titre.	Amount given intravenously. (c.c.)	Number of mice. Tested.	Number of mice. Survived.	Dilution of test spore suspension. (Dose = 0·05 c.c.)
F (<2·5 units antitoxin per c.c.)	1 : 500	0·25	15	15	
		0·10	15	15	
		0·05	5	5	10^{-5}
		0·025	5	4	
O	1 : 500	1·0	10	9	
		0·5	10	4	10^{-5}
		0·25	5	0	
Pure antitoxin (horse)	<1 : 10	10 units	10	0	10^{-5}
Normal rabbit	<1 : 10	0·25	10	0	10^{-5}
			5	2	10^{-6}

Observations on the animals in this experiment and those which follow were made for 14 days. A characteristic feature of infection with the "Pasteur" strain of *Vibrion septique* is the rapidity of death among the control animals. The mice receiving normal serum and those given "pure" antitoxin were found dead from a typical infection in 18–24 hours. In this and the later experiments death among mice receiving antibacterial serum was frequently delayed for 3–4 days; deaths occurring after this period were rare. The mice that received antibacterial or antitoxic serum were given at least one certain infecting dose (C.I.D.) of the spore + $CaCl_2$ mixture. Table I shows that 10 units of "pure" antitoxin failed to give any trace of protection, and since the F serum contained less than 2·5 units antitoxin per c.c., it is clear that the amounts of antitoxin given with the serum could play no significant part in the protection conferred. The titres of O antibody in the F and O sera are of the same order, but the protective value of the F serum is significantly greater than that of the "pure" O serum. This result clearly substantiates those of the earlier experiments (Henderson, 1935); and it remained to be shown by absorption experiments that the additional protective action was independent of the O antibody content of the serum.

THE EFFECT PRODUCED BY THE ABSORPTION OF O ANTIBODY ON THE PROTECTIVE VALUE OF F AND O SERA.

Portions of the F and O sera used for the previous experiment were absorbed with homologous O antigen. The possible effect of non-specific absorption was controlled by absorbing other portions of the same sera with a heterologous O antigen (*Vibrion septique*, strain "Milk") known to possess a highly specific O component. The technique of absorption was similar to that previously described (Henderson, 1934), and absorption effects were carefully controlled by agglutination tests. Thus, the sera absorbed with homologous O antigen were shown to have a titre of less than 1 in 5, whereas absorption with the heterologous antigen failed to reduce the titre appreciably. The F titre remained unchanged. For the in-vivo experiment groups of mice received graduated amounts of each serum sample, and all mice were tested with one C.I.D. of the homologous spore + $CaCl_2$ suspension. The results are summarized in Table II.

TABLE II.—*The Influence of Absorption of O Antibody on the Protective Value of F and O Sera.*

Type.	Serum. Absorbed with—	O agglutinin titre.	Amount given intravenously (c.c.).	Number of mice. Tested.	Survived.
F	Heterologous O suspension of *Vibrion septique*	1 : 500	0·100	10	10
			0·050	10	10
			0·025	10	9
	Homologous O suspension	<1 : 5	0·250	10	9
			0·100	10	10
			0·050	10	4
O	Heterologous O suspension	1 : 500	1·000	10	8
	Homologous O suspension	<1 : 5	1·000	10	0
Normal rabbit	Untreated	<1 : 5	1·000	13	0

Table II shows that absorption of the O serum with homologous O antigen removes all protective antibody. A similar absorption of the F serum reduces the protective value, but the serum remains much more efficient than the *unabsorbed* O serum. From the previous experiment it is known that antitoxin plays no significant part in the results obtained, and it would appear to be established, therefore, that *Vibrion septique* possesses, in addition to O antigen, a heat-labile antigen which stimulates the production in rabbits of a highly effective antibody against experimental infection in the mouse.

A comparison of the efficiency of F serum with that of "pure" antitoxin yielded some interesting quantitative data. An F serum was obtained from the rabbit by the method described above; the bacterial suspension from approximately 20 c.c. of culture served for the complete course of immunization, and the serum was available for use 21 days after the first injection of vaccine.

The " pure " antitoxin selected for comparison was a natural serum, prepared in the horse, which contained 100 international units per c.c. Groups of mice were injected intravenously with graduated amounts of each serum, and 24 hours later they were given one C.I.D. of an " activated " spore-suspension. The results, which are summarized in Table III, show that the F serum contained less than 5 units of antitoxin per c.c., the dose per mouse, therefore, being less than 0·1 unit. Moreover, 20 units of antitoxin were required to give approximately the same degree of protection as 0·02 c.c. of the antibacterial serum ; from this it follows that a natural serum containing not less than 1000 units of antitoxin per c.c. would be required to give an equivalent degree of protection. The choice of animal for the production of serum may, however, make these results more apparent than real, and it has yet to be shown that an F serum prepared in the horse is indistinguishable from an F serum prepared in the rabbit. Nevertheless, the ease of production of the F serum suggests that it may find a practical application as a prophylactic against infection with *Vibrion septique*.

TABLE III.—*The Relative Value of Antitoxic and Antibacterial Sera in the Control of an Experimental Infection.*

Type.	Serum. Units of antitoxin per. c.c.	Amount given intravenously.	Number of mice. Tested.	Survived.
Pure antitoxin (horse)	100	5 units	10	0
		10 ,,	10	0
		20 ,,	10	6
F	>2·5	0·02 c.c.	10	8
	<5·0	(<0·1 unit)		
Normal rabbit	<2·5	0·2 c.c.	10	0

THE TYPE SPECIFICITY OF THE F ANTIBODY.

Four highly specific O antigens are known to occur among the strains of *Vibrion septique* so far examined, and in-vivo experiments have shown that this specificity limits the protective value of a " pure " O serum to the homologous strain. Four H antigens are also known, but a common H factor is present in most of the strains, and for this reason H specificity is less sharply defined than that of O (Henderson, 1934). Further, the specificities of the H and O antigens do not necessarily run in parallel ; thus, strains occur which possess identical O but different H antigens.

In view of these facts it was of interest to examine F sera for the presence of type-specific protective antibodies to the labile components of the cell, and for this purpose cross-protection tests in mice were undertaken. Two type-specific F and two type-specific O sera were selected. These were first examined in cross-agglutination tests, and were then titrated in mice against the homologous and heterologous strains used in their production. The results are recorded in Table IV.

TABLE IV.—*The Type Specificity of the F and O Antibodies.*

Serum.	Agglutinin content. Type.	Antigen.	Titre.	Infecting suspension.	Amount of serum (c.c.).	Mice. Tested.	Survived.
10¼ + 11 III ("Pasteur" F)	O	"Pasteur"	2000	"Pasteur"	0·050	6	6
		"Milk"	<20	„	0·025	6	5
				„	0·010	6	1
	F	"Pasteur"	200,000	"Milk"	1·000	4	4
		"Milk"	2000	„	0·500	4	1
12 + 13 II ("Milk" F)	O	„	1000	„	0·250	6	6
		"Pasteur"	<20	„	0·100	6	3
				„	0·050	6	0
	F	"Milk"	5000	"Pasteur"	1·000	4	0
		"Pasteur"	200	„	0·500	4	0
1 + 2 III ("Pasteur" O)	O	„	1000	„	0·500	6	6
				„	0·250	6	4
		"Milk"	<20	„	0·100	6	0
				"Milk"	1·000	4	0
3 + 4 III ("Milk" O)	O	„	500	„	0·500	6	6
				„	0·250	6	2
		"Pasteur"	<20	"Pasteur"	0·100	6	0
					1·000	4	0

From the records shown in Table IV of the agglutinin content of the sera it is seen that the O antibody in each case has a high degree of specificity, and that the overlapping exhibited by the F antibody is distinct but of a low order. A titration of the infecting suspensions in mice receiving normal rabbit serum showed that the test dose of each suspension contained 5–10 C.I.D. The serum 10 + 11 III contained approximately 2·5 units of antitoxin per c.c., and the serum 12 + 13 II had less than 0·5 unit per c.c. None of 6 mice each receiving 10 units of antitoxin survived the test dose, and it may be concluded that antitoxin played no part in the test. The following points emerge from the results: (1) There is a close relationship between the in-vitro and in-vivo findings; (2) the strict specificity of O antibody observed in the cross-agglutination tests is sharply reflected in the cross-protection tests; (3) the type-specificity of F antibody is also noted in protection; when the homologous F titre is low no cross-protection occurs, but when the homologous F titre is high and the degree of overlapping with the heterologous strain is appreciable, evidence of protection due to heterologous F antibody is detected; (4) the higher degree of protection given by an F + O serum than by a "pure" O serum is clearly demonstrated; and (5) the F agglutinin titre gives some index of the protective value of the serum against infection with the homologous strain.

THE NATURE OF THE F ANTIGEN.

The discrimination of various types of agglutination among the spore-bearing anaërobes presents considerable difficulties. It has been noted (Henderson, 1932) that H and O agglutinins cannot readily or accurately be distinguished in the same test-tube, and that the H titre of a serum should be gauged only after complete absorption of the O antibody. In the experiments

described above there is no doubt but that the so-called F agglutination would normally be interpreted as indistinguishable from the H type of aggregation described by Felix and Robertson (1928). Since there appeared to be a correlation between the F agglutinin titre and the protective-antibody value of an O-absorbed F serum, the possible role in protection of the so-called H antibody had to be considered. The categorical statements concerning the function of H antibodies in general appeared at first sight to make it improbable that the H antibody could serve as a protective agent. Nevertheless, it may be stated now that although the experiments designed to solve this problem have not given indisputable proof that H antibody plays a part in protection, the evidence obtained indicates that an antibody indistinguishable from it does actually confer a high degree of protection against an experimental infection.

If it be true that H antigen is represented by the flagella of the organism, the problem might readily have been solved by working with a non-motile strain of *Vibrion septique*. Such a strain, unfortunately, is not available, and less direct methods of approach had to be adopted. In the first instance, it was considered possible that *Vibrion septique* had a labile somatic antigen similar to the so-called virulence antigen (Vi) of *S. typhi* recently described by Felix *et al.* (1934), but that the H type of agglutination encountered masked the occurrence of aggregation produced by a labile somatic component and its corresponding antibody. If such an antibody were present in F serum it might act in parallel with H antibody and thus lead to a false interpretation of the results.

The Vi antigen of *S. typhi* and other members of the Salmonella group has been shown by Felix and Pitt (1936) to resist the action of alcohol, in contrast to the H antigen, which is destroyed by similar treatment. It is also known that the so-called H antigen of *Vibrion septique* is destroyed by alcohol (Felix and Robertson, 1928), and attempts were therefore made to demonstrate the presence of a second labile antigen by employing this method.

The Preparation of an Alcohol-treated Suspension.

Growth deposits were obtained as in the preparation of F antigen. A suspension of the washed organisms in distilled water with an opacity 10×25 (Brown's scale) was diluted with 96 p.c. ethyl alcohol to opacity 4–5. The container with glass beads was stored at room temperature for 48 hours and repeatedly shaken. The suspension was then washed three times in a large volume of distilled water and finally resuspended in distilled water to the required density. A freshly prepared suspension was used for each experiment.

A serum was obtained by immunizing rabbits with F suspension prepared from the strain "Pasteur", and portions of it were absorbed with—(*a*) alcohol-treated suspension, (*b*) formolized suspension, and (*c*) O antigen. These antigens were prepared from the same suspension of the homologous strain and an equal amount of each was used for the absorption. The absorbed serum samples together with the untreated serum were tested *in vitro* with F and O suspensions prepared from the homologous strain and then titrated in mice. Table V summarizes the results.

TABLE V.—*Absorption Test.*

Immune rabbit serum. (< 0·5 unit antitoxin per c.c.)	Agglutinin titre. H.	Agglutinin titre. O.	Amount of serum given intravenously (c.c.).	Mice. Tested.	Mice. Survived.
Unabsorbed	100,000	5000	0·10	10	10
			0·05	10	8
			0·02	10	7
O absorbed	100,000	<1 : 2	0·20	10	8
			0·10	10	4
			0·05	10	4
Absorbed with alcohol-treated suspension	50,000	<1 : 2	0·20	10	6
			0·10	10	4
			0·05	10	2
Absorbed with formolized suspension	500	<1 : 2	0·20	10	0
Normal rabbit serum	0	<1 : 2	0·20	10	0
Pure antitoxin (horse)	..	..	20 units	10	0

Absorption with all three suspensions reduced the O titre to less than 1 in 2, but the resultant titres of H antibody varied according to the antigen used. The absorbed sera were also tested in agglutination with alcohol-treated suspension. The influence of alcohol treatment on flagellar substance was shown by the almost complete absence of H agglutination with the serum portions absorbed respectively with O antigen and alcohol-treated suspension. On the other hand, no evidence of a third type of agglutinin of the nature of Vi antibody could be detected by this means.

For the protection test the infecting suspension was prepared from the homologous strain. All mice received at least one C.I.D. of a spore + $CaCl_2$ mixture. The role of antitoxin was carefully controlled ; the F serum contained less than 0·5 unit of antitoxin per c.c., and 10 units of "pure" antitoxin failed to protect mice against the test dose. It is seen from Table V that the complete absorption of O antibody reduced the protective value by approximately 50 p.c., and that the protective action was abolished when both O and H antibodies had been removed with F suspension. The protective value of the serum absorbed with an alcohol-treated suspension is about the same as that of the O-absorbed sample ; the slightly lower value recorded by the former is actually in agreement with the fact that the H titre had been reduced. From this evidence it is necessary to conclude, either that H agglutinin and the associated protective antibody are identical, or that *Vibrion septique* possesses a labile cell component distinct from H agglutinogen, which, unlike the Vi antigen of *S. typhi*, is destroyed by treatment with alcohol.

The examination of serum prepared by immunizing rabbits with alcohol-treated suspension (A serum) has failed to give evidence for a second labile antigen. Table VI summarizes the results obtained with such a serum before and after absorption of O antibody ; a homologous F serum was included in the test for comparison. The O agglutinin titre of the A serum is shown to be approximately 4 times higher than that of the F serum, whereas its H titre is approximately 100 times lower. Complete absorption of O antibody,

however, reduced the protective value of the F serum by 50–70 p.c.; the similarly treated A serum, used in large excess, failed to protect mice.

TABLE VI.—*O Absorption of F and A Sera.*

Type of serum.	Serum treatment.	Agglutinin titre. H.	Agglutinin titre. O.	Amount given intravenously (c.c.)	Mice. Tested.	Mice. Survived.
F	Unabsorbed	100,000	5000	0·05	10	8
				0·02	10	2
	O absorbed	100,000	<1 : 10	0·20	10	9
				0·10	10	5
				0·05	10	1
A	Unabsorbed	1000	20,000	0·05	10	9
				0·02	10	1
	O absorbed	1000	1 : 20	0·50	10	0
Normal rabbit	Untreated	..	..	0·50	10	0

From the experiments on the type specificity of F antigen it appears that this specificity limits the protective value of F antibody to the homologous strain; the results of one such test are summarized in Table IV. The strains examined, however, had specific O antigens in addition to the specific F factors, and for this reason it was not known whether the protective antibody in an O-absorbed F serum was associated with a specific labile somatic component or with the specific labile H antigen. It was considered, therefore, that the evidence implicating the so-called H antigen might be examined by cross-protection tests with F sera prepared by immunizing with strains possessing identical O antigen but specific H factors. By this means it would be possible to absorb a specific F serum with an unheated suspension of the heterologous strain, thereby leaving the specific H agglutinin but removing, in addition to the common O antibody, any protective substances common to both strains and associated with hypothetical labile somatic components of the cell. Such strains have already been described (Henderson, 1934), and for the present experiments an F serum prepared against one of them (" Milk ") was selected as having a low H agglutinin titre with a high degree of specificity, and an F serum for the other strain (" Jerral ") was chosen with a high specific O titre, but showing also a considerable degree of cross H agglutination with the heterologous strain, the cross H agglutinin owing its presence to the common H factor, already mentioned, which is shared by all strains of *Vibrion septique*. Portions of the " Milk " F serum were tested before and after absorption with O antigen, and similarly, portions of the " Jerral " F serum with O antigen and with F suspension from the heterologous strain. Absorption effects were recorded by in-vitro tests, and the sera were then tested in mice against infection with the homologous and heterologous strains. The test inoculum of each infecting suspension contained at least 1 C.I.D., and 10 units of " pure " antitoxin failed to protect control mice. The results are summarized in Table VII.

Table VII shows that the unabsorbed sera gave some degree of protection against infection with the heterologous strain, but only in amounts greatly in excess of that required to protect against infection with the homologous strain.

TABLE VII.—*Cross-Protection Experiments.*

Serum.	Serum treatment.	Agglutinin titres. Homologous. H.	Homologous. O.	Heterologous. H.	Heterologous. O.	Test spore suspension.	Amount of serum given (c.c.).	Mice. Tested.	Mice. Survived.
"Milk" F	Unabsorbed	20,000	1000	500	1000	"Milk"	0·10	10	7
							0·05	10	5
							0·02	10	1
						"Jerral"	0·50	10	9
							0·20	10	3
	O absorbed	20,000	<1 : 10	500	<1 : 10	"Milk"	0·20	10	8
							0·10	10	2
						"Jerral"	0·45	10	0
"Jerral" F	Unabsorbed	100,000	500	5000	500	„	0·05	10	9
							0·02	10	4
							0·01	10	2
						"Milk"	0·20	10	8
							0·10	10	3
	O absorbed	100,000	<1 : 10	5000	<1 : 10	"Jerral"	0·10	10	10
							0·05	10	7
						"Milk"	0·50	10	7
							0·20	10	6
							0·10	10	0
	Absorbed with heterologous ("Milk") F suspension	50,000	<1 : 10	<1 : 50	<1 : 10	"Jerral"	0·10	10	7
							0·05	10	5
						"Milk"	0·50	10	0

Absorption of the O antibody from the serum with a low H agglutinin titre (Milk F) simultaneously removed all protective antibody for the heterologous strain, whereas, for the homologous strain the value was only slightly reduced—a result which shows that the cross-protective value of the unabsorbed serum was due entirely to the O antibody content. When, however, the serum (Jerral F) which exhibited a considerable degree of cross H agglutination was absorbed with O antigen it retained some protective antibody for the heterologous strain. On the other hand, simultaneously with the removal of O antibody and the H agglutinin common to the heterologous strain by absorption with F heterologous suspension, all cross-protection vanished ; the same serum when tested against infection with the homologous strain gave a value similar to that of the O-absorbed portion. It appears, therefore, that the specificity in protection shown by F antibody is paralleled solely by the specificity of the H agglutinin content of the serum, but it may still be postulated that the two are not identical. For example, it might be argued that the strains examined possess, in addition to specific H antigen, labile somatic components, the specificity of which bear no relationship to their O antigens, which are identical but closely parallel that of the H factors. Under such circumstances the conclusion that H agglutinin and F antibody are identical would be a false one. There is no available evidence, however, of an F antigen distinct from the H substance, and the close parallelism between the behaviour of the F fraction and the known character of the H antigen justifies the provisional conclusion that they are identical.

DISCUSSION.

The researches of Weil and Felix (1916) concerning the agglutination reactions of *Bact. proteus* with the serum of typhus fever patients laid the foundation of our present knowledge concerning the function of flagellar and somatic antigens and antibodies. The hypothesis that motile organisms possess an antigen referable to the flagellum, however, would appear to date from the early observations of Smith and Reagh (1903) and Beyer and Reagh (1904) on "*Bacillus cholerœ suis*" and of Joos (1903) on *S. typhi*. Support for this view, which is now generally accepted, has been given mainly by the work of Orcutt (1924), Yokota (1925) and Arkwright (1927*a*). The association of flagellar antigen with the labile H antigen of Weil and Felix (1917) has been demonstrated by many workers, including Joetten (1919), Braun and Schaffer (1919), White (1925), and Felix and Robertson (1928).

The consensus of opinion concerning the immunological significance of H antigen and antibody in infective processes is expressed by Topley (1933) when he writes that "... the flagellar antigens and the corresponding antibodies play little if any part in the mechanism of specific antibacterial immunity ...". This conclusion has been based on the "double receptor" hypothesis of Weil and Felix (1917), and the evidence in its favour has accrued from a pursuit of the method of "qualitative receptor" analysis introduced by these authors to explain the serological behaviour of strains of *Bact. proteus*. Thus, Weil and Felix (1920), Weil (1921), and Felix and Robertson (1928) showed by their technique that H antibody played no

significant role in complement-fixation. From a study of the bactericidal property of immune serum several workers, including Felix and Olitzki (1926), have shown that H antigen does not stimulate the production of bactericidal immune body, and Braun and Nodake (1924) and Bhatnagar (1935) have shown that the H antibody of *S. typhi* has no appreciable influence on phagocytosis. Felix (1924) reported the results of clinical observation on typhoid patients and the analysis of their serum reactions, and concluded that H antibody had no immunological significance. The negative role of H antigen in the production of active immunity against experimental infection with members of the Salmonella group has been described by Arkwright (1927*b*), and Greenwood, Topley and Wilson (1931). Felix and Pitt (1934) conclude that the function of H antibody in protection " need not be considered in this connection, since it is well known that H antibodies are negligible as protective agents ". These opinions, however, are traversed to a greater or less degree by the work of White (1926), Hofmeier (1927), Craigie (1931) and Abdoosh (1936) concerning the function of the H antibody of *S. typhi* in complement-fixation ; that of Springut (1927) on protection against infection ; and of Mudd, Lucke and Strumia (1933) on bacteriotropic action. At least two reasons for this discordance can readily be traced, the one to differences in technique, and the other to the existence in certain organisms of labile antigenic constituents, exclusive of the flagellar substance, that have hitherto been unrecognized. With regard to technique, the better appreciation of the physio-chemical conditions governing antigen-antibody reactions will eventually remove differences of opinion arising from in-vitro testing ; and especially is this true for the complement-fixation reaction. The second explanation has assumed considerable importance since the demonstration of the heat-labile Vi antigen in certain strains of *S. typhi* (Felix and Pitt, 1934). Felix and his colleagues have shown that this antigen stimulates the production of an antibody responsible for active and passive protection and capable of functioning *in vitro* as agglutinin, precipitin and bacteriotropin, thus fulfilling the requirements previously ascribed by the " double receptor " hypothesis only to the heat-stable somatic antigen. With this evidence in mind it may be possible, in part at least, to explain the discordant results obtained by various workers. Thus, for example, the complement-fixing power occasionally attributed to the H antigen of *S. typhi* may be explained by the presence of a Vi component in the reagents ; under these circumstances the flagellar substance may wrongly be invested with the power to bind complement. On the other hand, it is a curious coincidence that those workers who have denied the power of the heat-labile antigen to fix complement did not encounter this phenomenon, at least with *S. typhi*, by the unwitting use of Vi strains and sera. In active and passive protection experiments also, the value of labile antigen would be determined by the presence or absence of the Vi component. For example, Felix and Pitt (1935) have pointed out that in Arkwright's experiments on active immunization (1927) the absence of protection by a rough motile variant of *S. typhi* was probably due to the fact that Vi antigen was lacking from the strain he employed. Again, in the experiments on active immunization described by Greenwood, Topley and Wilson (1931) the formalin and heat treatment used in the preparation of the *S. aertrycke* antigens

might have been sufficient to destroy the prophylactic value of any Vi-like component.

These observations are of immediate interest in the interpretation of the results presented in this paper. In the first place it is clear that the lax use of the term " H antigen " to include all the heat-labile cell components should now cease ; this name should be applied solely to the flagellar substance.

A theoretical argument that has been advanced to explain the peculiar ineffectiveness of H antibody in protection is that it merely immobilizes the organism, and in no way sensitizes the all-important surface antigens which prepare the way for phagocytosis and lysis. Immobilization may, however, be ineffective because organisms transported by the body fluids to other foci are still capable of multiplication in the new surroundings. This may be true for the typical aërobic pathogen, but for the anaërobe a different explanation is needed. Here, a lowered oxygen tension of the tissues at the site of infection is essential not only for germination of spores, but for the maintenance of actively dividing vegetative forms. The accumulation of the by-products of growth which rapidly follows the initiation of infection tends to check active bacterial proliferation, unless the original region of lowered oxygen tension is increased by the elaboration of a necrotizing type of toxin. Bacterial invasion extends only to the margin of the damaged tissue ; spores and even vegetative forms transported therefrom to normal tissues remain dormant unless accompanied by depressants of oxygen tension. The process of invasion with a pathogenic anaërobe, therefore, differs fundamentally from that of an aërobe, and for this reason it is not improbable that an immobilizing agent such as flagellar antibody may assist in checking the advance and migration of the invader within the infected region. On this hypothesis it is not necessary to postulate that flagellar or H antibody. if effective in protection, should manifest itself *in vitro* by the fixation of complement or as a bacteriotropin. The work described in the foregoing pages was begun with these considerations in mind.

The first two experiments described in the text clearly demonstrate that an antibody intimately associated with the labile constituents of the cell plays an important role in passive protection ; the results of the agglutination test show that only one type of antibody, namely flagellar agglutinin, can be associated with this protective element. It is difficult to reconcile this result with that reported by Robertson and Felix (1930). These authors examined the protective action of antibacterial serum in the control of infection with *Vibrion septique*, and concluded that the so-called H antibody plays no part in determining the value of a serum. This conclusion, however, is based only on a few experiments with two sera containing H agglutinins and no satisfactory explanation of the result can be offered.

The demonstration by Felix and his colleagues of a labile somatic antigen in *S. typhi*, however, indicated the possibility of the existence of a similar type of antigen in *Vibrion septique*, and the subsequent experiments described in the present paper were devised with the object of demonstrating the presence of such an antigen. It has already been mentioned that the technical difficulties involved in agglutination tests with the spore-bearing anaërobes make the possibility of detecting a third type of agglutinin much more remote than might be expected from Felix's experience with *S. typhi* ; and, indeed, all

efforts in this direction failed. The problem might readily have been solved by working with a non-motile strain of *Vibrion septique*, but such a strain, unfortunately, is not available. Experiments were therefore devised to demonstrate the influence of destruction of the flagellar substance on the prophylactic value of vaccines, and also on the power of such suspensions to absorb from an F serum that protective portion associated with labile cell components.

It is known that treatment with ethyl alcohol destroys the flagellar antigen of *Vibrion septique* (Felix and Robertson, 1928). This agent was selected for the preparation of modified antigen because Felix and Pitt have recently shown that the Vi antigen of *S. typhi* is immunologically active after exposure to it. The results of experiments of this kind were conclusive; the in-vitro demonstration of a third type of agglutinin failed, so that the heat-labile flagellar antibody was the only one that could be associated with the protective element of a serum from which O antibody had been removed. Experiments with other types of modified antigen were not undertaken. The absence of a frank in-vitro manifestation of a third antigen and the absence also of any knowledge concerning the chemical constitution of the antigen involved made it appear that the results of any experiments with modified antigens could be interpreted in either of two ways: If the evidence should point to flagellar substance as the antigen in question, the obvious criticism would be that the treatment of the bacterial cell had destroyed a second labile factor. On the other hand, if a serum with a potent flagellar agglutinin was shown to have no protective value in the absence of O antibody, it could be argued that the functional activity of the flagellar substance had been modified, the alteration affecting solely its power to stimulate the production of protective antibody, and leaving its agglutinogenic ability unimpaired. Evidence in favour of the latter alternative has already been produced by Avery and Goebel (1933) in their experiments with the acetylated and de-acetylated polysaccharide of pneumococcus (Type I), and by Felix and Bhatnagar (1935), who have shown that the treatment of the Vi antigen of *S. typhi* with formalin has a deleterious action on its prophylactic value but does not impair the agglutinogenic activity.

The specificity of flagellar antigen exhibited by different strains of *Vibrion septique* afforded the opportunity of examining further, by cross-protection tests, the association of flagellar agglutinin with the protective antibody in question. Preliminary tests showed that the specificity in protection ran parallel with that of flagellar agglutinin, and the agglutinin titre gave some indication of the protective value to be expected. Finally, the examination of sera prepared by immunizing with strains possessing identical heat-stable antigens and specific flagellar antigens established the fact that the protective antibody associated with the labile antigen bore no relationship in specificity to the somatic (O) antibodies; the protective value was again determined by the degree of specificity shown by the flagellar agglutinin for any particular strain. This result lends strong support to the preceding evidence that the flagellar antigen of this organism is intimately associated with the production in rabbits of an antibody highly efficient in protection against experimental infection.

SUMMARY.

A heat-labile bacterial antigen of *Vibrion septique* has been shown to stimulate the production in rabbits of an antibody which controls experimental infection in mice. The experimental evidence points to the association and perhaps the identity of this antigen with the flagellar substance. On this theory the antigen is the H antigen and the protective antibody the H antibody.

The relative value of an antitoxic and an antibacterial serum has been examined and the practical application of an antibacterial prophylaxis has been indicated.

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STUDIES IN DIPHTHERIA TOXIN PRODUCTION. III: A SIMPLE GELATIN HYDROLYSATE MEDIUM AND SOME PROPERTIES OF THE TOXIN PRODUCED THEREON.

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IN the first two papers of this series (Pappenheimer and Johnson, 1936; Pappenheimer, 1936) it was shown that the yield of diphtheria toxin in a medium supporting growth of the diphtheria bacillus was highly dependent on the concentration of minute amounts of inorganic iron salts. It was demonstrated that when the iron concentration was suitably controlled, potent toxin was produced on a simple amino-acid medium similar to that described by Mueller (1935*a*). This amino-acid medium, however, has not proved practical for preparing toxin in large quantity. In the first place, the amino-acids necessary are extremely costly; secondly, the liver fraction essential to provide accessory growth factors is not readily available in most laboratories; and finally, the optimum balance of traces of inorganic constituents has not been indicated. We are now describing a medium for the production of diphtheria toxin which has a complete acid hydrolysate of gelatin as a base, supplemented by small amounts of methionine, cystine and tryptophan, salts and alcohol-soluble accessory growth factors contained in either purified yeast or liver extract. To date we have prepared more than 300 l. of this medium, which has produced an average of more than 30 Lf of toxin per c.c. The cost of preparing the medium is less than one-half that of the amino-acid medium and is not greater than the cost of veal infusion medium.

EXPERIMENTAL.

Preparation of Gelatin Hydrolysate Medium.

dl-methionine	4 g.	Liver or yeast extract preparation	100 c.c.
dl-cystine	4 ,,	Gelatin hydrolysate	500 ,,
l-tryptophan	1·5 ,,	Distilled water to	20 l.
K_2HPO_4	40 ,,	Sugar + lactate solution (added separately before inoculation)	400 c.c.
NaCl	80 ,,		
$MgSO_4$	6 ,,		
$CaCl_2$ (33 p.c. solution)	6 c.c.		
$CuSO_4.5H_2O$	0·1 g.		

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The methionine, dipotassium phosphate and sodium chloride are placed in a 6-l. Pyrex Florence flask. 500 c.c. of stock acid hydrolysate of gelatin (note 1), 100 c.c. of purified liver or yeast extract preparation (note 2) and about 2 l. of distilled water are then added. The cystine is dissolved separately in the least amount of 1 : 1 hydrochloric acid (note 3) and added to the main lot, which is now made up to 10 l. with distilled water, distributed in three 6-l. Pyrex flasks and brought to pH 7·8–8·0 with 5*N* NaOH. 2 c.c. of 33 p.c. calcium chloride solution is then added to each flask and the medium boiled gently for 10 minutes (note 4). After it has been allowed to cool for a short time, it is filtered into a Pyrex 5-gallon carboy, the tryptophan, magnesium sulphate and copper sulphate are added (note 5) and it is made up to 20 l. Finally the reaction is readjusted, if necessary, to pH 7·6–7·8, and the slightly yellow medium distributed into Fernbach flasks (note 6), 600 c.c. to each flask, and autoclaved 15 minutes at 120° C. Before inoculation, 12 c.c. of a sterile solution containing 37 c.c. Merck's c.p. sodium lactate, 15 p.c. Merck's purified maltose, 7·5 p.c. Merck's c.p. dextrose (note 7) and 0·3 p.c. calcium chloride (note 8), are added to each flask, which is then inoculated with a 24-hour Park No. 8 culture (note 9). The flasks are allowed to incubate for 6 days at 35° C., after which the toxin is collected. The yield should be 30–40 Lf per c.c. (note 10).

NOTES.

1. The stock gelatin hydrolysate is prepared as follows: 1000 g. of Eastman's "de-ashed" gelatin is treated with 5 l. of 1 : 1 hydrochloric acid and autoclaved 3 hours at 123° C. The deeply coloured hydrolysate is evaporated to a thick syrup *in vacuo*, and, after distilled water has been added, again evaporated under reduced pressure. The black syrup is dissolved in about 2 l. of water and adsorbed with 40–50 g. of decolorizing charcoal. The clear filtrate is made up to 2500 c.c. for stock and is sufficient for 100 l. of medium.

2. The purified liver preparation is made according to Mueller (1935*b*). 1 c.c. is equivalent to 10 g. of liver.

The purified yeast fraction may be prepared as follows: 1000 g. of marmite (vegex) is dissolved in 2·5 l. of distilled water, and a saturated solution of basic lead acetate added until there is no further precipitation. The precipitate is removed and the filtrate is acidified and saturated with hydrogen sulphide. After the lead sulphide has been removed, the filtrate is concentrated *in vacuo* to a small volume (200–300 c.c.) and 5 volumes of alcohol are added. The insoluble material is filtered off, the filtrate concentrated to a syrup *in vacuo* and the water-soluble residue made up to 1 l. with distilled water. 5–10 c.c. of this solution per l. of medium are sufficient for maximum growth and toxin production. The yeast fraction may be further purified without altering the yield of toxin.

3. Cystine is dissolved separately with HCl, since it otherwise goes into solution only with great difficulty.

4. The purpose of the calcium phosphate precipitation at pH 7·8 is to remove excess iron by adsorption. If all the iron is removed at this point the proper concentration for toxin production may be adjusted later. (See note 7.)

5. Since tryptophan is rapidly destroyed in acid solution, it is not added until after the solution has been neutralized. Magnesium sulphate is not added until this stage in order that none of it may be carried down with the precipitated calcium phosphate.

6. Fernbach flasks made of soft glass (Pappenheimer and Johnson, 1936) have proved more satisfactory than those of Pyrex with this medium, particularly if the medium is allowed to incubate for several days before inoculation. However, 25–30 Lf per c.c. of toxin may be produced in Pyrex if soft glass flasks are not available.

7. The iron necessary for toxin production (see note 4) is added as impurity in the sodium lactate + sugar solution. The grade of reagents described fortunately contains close to the optimum

amount of iron. Should any trouble arise on this score, however, it may be necessary to remove iron from the sugars by the calcium phosphate method at pH 7·8, or from the sodium lactate by the use of re-distilled lactic acid, and then determine the quantity of any iron salt which it is necessary to add in order to obtain the highest yield of toxin. The reagents may be tested for their iron content with dipyridine or thiocyanate. The optimum iron content of the final medium should be approximately 0·05 mg. per l.

8. Since almost all the calcium previously added has been precipitated as phosphate, a further quantity is added with the lactate sugar solution.

9. The strain of organism is of great importance. Park No. 8 strains vary widely both in toxin-producing capacity and growth requirements, and it is advisable to test a number of Park No. 8 strains to determine the best available.

10. Toxin containing 30–40 Lf may be obtained regularly in soft glass Fernbach flasks. Choice of a diphtheria strain of low toxicogenicity, the use of other types of glassware, an unfavourable iron concentration or an unfavourable surface/volume ratio are some of the factors which may decrease this yield. For maximum yields and reproducibility, care must be taken to use clean Pyrex vessels throughout the preparation of the medium.

ATTEMPTS TO IDENTIFY THE STIMULATING ELEMENT IN SOFT GLASS.

In the previous communication it was noted that medium autoclaved in certain soft glass Fernbach flasks available in this laboratory gave a considerably higher yield of toxin than the same medium in Pyrex. Spectroscopic analyses have now been made of the gelatin hydrolysate medium (without added copper sulphate or the sugar + lactate solution) autoclaved in soft glass and in Pyrex. We are greatly indebted to Mr. David Richardson of the Massachusetts Institute of Technology for carrying out these analyses. Table I, drawn up by Mr. Richardson, compares the inorganic composition of media autoclaved in the two types of glassware.

TABLE I.—*Inorganic Constituents of Gelatin Hydrolysate Medium Autoclaved in Soft Glass and Pyrex Fernbach Flasks.*

Element.	Pyrex.	Soft glass.	Element.	Pyrex.	Soft glass.
Aluminium	1	1	Phosphorus*	2	2
Boron	2	2	Potassium*	2	2
Calcium*	0·97	1·35	Silicon	2	2
Copper	0·12	0·20	Sodium*	3	3
Iron	1	1	Strontium	0·01	0·05
Lead	1	1			(estimate)
Magnesium*	3	3	Titanium	1	1
Manganese	0	0	Zinc	1	0
Nickel	1	1			

0 = Elements not detected, in addition to those not listed. 1 = Spectroscopic trace, probably less than one part per million. 2 = Minor constituent, less than 1 p.c. 3 = Major constituent. (Quantitative results are underlined and given in parts per million.)
* = Added during preparation of the medium.

Table I shows that the copper, calcium and strontium content of the medium is increased by autoclaving in soft glass. Although the addition of calcium and copper salts in small quantity has definitely increased the toxin yield in Pyrex (from about 20 Lf per c.c. to 27 Lf per c.c.), we are still unable to duplicate

with Pyrex the high yields obtained in soft glass. Addition of strontium salts has no effect on the yield if further calcium is added with the sugar + lactate solution after removal of the calcium phosphate precipitate. Attempts to increase the yield in Pyrex by addition of traces of iron have also failed.

TOXOID FORMATION.

Table II shows the time necessary for complete detoxification of toxin produced on the gelatin hydrolysate medium with varying amounts of formalin.

TABLE II.—*Toxoid Formation with Varying Amounts of Formalin.*

P.c. formalin added.	Time for complete detoxification (days).
0·05	>127
0·10	95
0·15	41
0·20	27
0·25	17

Immunity tests with guinea-pigs have indicated that the toxoid made from toxin produced on gelatin hydrolysate medium is fully as effective an immunizing agent as that produced on other types of media.

PROPERTIES OF TOXIN PRODUCED ON SIMPLE MEDIA.

The gelatin hydrolysate medium gives no precipitate, before inoculation, with ammonium sulphate at any concentration. It follows that any precipitate obtained with ammonium sulphate after growth has taken place must be due to the metabolic products of the organism. The medium, therefore, should be well suited for the isolation of diphtheria toxin in a state approaching purity.

In some preliminary experiments, two lots of gelatin hydrolysate medium were inoculated and incubated. The first contained 10 mg. ferrous sulphate per l. and therefore produced no toxin. The second lot contained no added iron and yielded 35 Lf per c.c. Both lots were concentrated to 1/5 their original volume, *in vacuo* below 35° C., and ammonium sulphate added to 1/3 saturation. A precipitate formed in approximately equal amount in each lot. This precipitate was essentially atoxic and proved, by precipitin test against an anti-diphtherial protein rabbit serum, to be the same protein in each case. When a further quantity of ammonium sulphate was added to the filtrates to 2/3 saturation, a slight precipitate formed in the lot containing no added iron. Since the latter contained practically all the original toxin, it was surmised that the main bulk of the precipitate represented the actual toxin itself. Repeated fractionation with ammonium sulphate, followed by adsorption of certain coloured impurities and inactive protein with aluminium hydroxide cream and finally by dialysis (all at a pH kept above 6·0), gave highly pure preparations of toxin. The purest so far obtained are pale yellow in 2 p.c. solution (*ca.* 6000 Lf/c.c.), and contain 0·00046–0·00047 mg. nitrogen per Lf unit and 80,000 M.L.D. per mg. of nitrogen. This is in close agreement with

the purest preparations obtained by Eaton (1936) from a peptone medium. His best figures lie between 0·0005 and 0·0006 mg. nitrogen per Lf, of which 0·00045–0·0005 mg. nitrogen is precipitable by trichloracetic acid. This nitrogen value per Lf unit for pure toxin is also confirmed by results of a quantitative study of the flocculation reaction (Pappenheimer and Robinson, 1937). This study utilized a method which was not dependent on the purity of the toxin employed, and indicated 0·00046 mg. nitrogen per Lf unit of diphtheria toxin. Like Eaton's, our toxin of highest purity behaves as a typical protein which is very sensitive to denaturation on the acid side of pH 6. The purification and chemical characterization of diphtheria toxin will be considered in detail elsewhere.

If toxic diphtheria filtrates from amino-acid or gelatin hydrolysate media are acidified with acetic acid and extracted with ether, the porphyrin described by Coulter and Stone (1931) and by Wadsworth, Crowe and Smith (1935) passes into the ether layer. Atoxic filtrates from organisms grown in the presence of 1 : 500,000 iron do not produce this porphyrin. This is in keeping with Coulter and Stone's observation that the porphyrin produced is proportional to the yield of toxin.

DISCUSSION.

Protein hydrolysates have been used by several workers as a basal medium for the growth of pathogenic bacteria. Mueller *et al.* (1933) have used an acid hydrolysate of casein for growing a number of organisms, including *C. diphtheriæ*; and Knight and Fildes (1933), and Knight (1935), have employed an acid hydrolysate of gelatin for the growth of *Cl. sporogenes*, *Staphylococcus* and other bacteria. We have preferred to use gelatin in the present instance, since the Eastman product has a very low ash content and the hydrolysate contains a minimum amount of unknown material.

It has just been reported by Mueller (1937) that pimelic acid in minute traces is essential for growth of the diphtheria bacillus, and it is hoped that the liver or yeast fraction may be dispensed with entirely as soon as the nature of the remaining factor or factors which they contain has been elucidated.

If the precautions noted in the experimental part of this paper are observed, the gelatin hydrolysate medium possesses several advantages over media hitherto employed in the preparation of diphtheria toxin: (*a*) It is simple and easy to make up. (*b*) The yields are high and reproducible in spite of the very low nitrogen content (1·8 mg. per c.c.). (*c*) The toxin is completely converted to toxoid with only 0·2 p.c. formalin in 4 to 5 weeks. (With veal infusion medium and 0·35 p.c. formalin toxoid formation often requires several months to go to completion.) (*d*) Finally, since the gelatin hydrolysate medium contains insignificant amounts of material of high molecular weight, highly purified and concentrated toxin may be obtained by simple ammonium sulphate fractionation and dialysis.

There are two points, however, which should be emphasized once more, namely the strain of organism used and its care. Dr. J. H. Mueller informs us that Park No. 8 strains obtained from various sources differ greatly in their growth requirements, and in our previous paper we showed that of five Park

No. 8 strains tested on the amino-acid medium, no two possessed the same toxin-producing capacity. The Albany No. 5 strain of Park No. 8 which we have used in this work has consistently produced potent toxin on all the usual media. This capacity, however, may be seriously impaired if the Albany culture is given repeated daily transplants on Wadsworth and Wheeler (1934) peptone medium at 37° C. Such loss in toxicogenicity when the organism is subcultured at incubator temperature has occurred too frequently to permit a doubt that it is anything but a real phenomenon. Cultures transferred on peptone medium two or three times a week at room temperature have maintained their capacity for toxin production unimpaired for the past eighteen months.

Since small amounts of iron are known to inhibit toxin production, the question arises as to whether the organism is permanently affected by this treatment. Thirty-eight successive subcultures of the Albany strain on peptone medium containing 1 part in 500,000 of added inorganic iron have failed to reduce its toxin-producing capacity when transferred to a medium to which iron has not been added.

SUMMARY.

1. A simple gelatin hydrolysate medium of low nitrogen content (1·8 mg./c.c.) for the production of diphtheria toxin of high titre is described. Some of the advantages of this medium over those usually employed are discussed.

2. Preliminary experiments on the purification of toxin prepared on gelatin hydrolysate medium are reported.

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THE COMBINING PROPERTIES OF VACCINIA VIRUS WITH THE ANTIBODIES DEMONSTRABLE IN ANTI-VACCINIAL SERUM.

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DURING recent years considerable advances have been made in the study of the antigenic composition of vaccinia virus. Reviews of these advances are available in papers by Craigie (1935), and Parker and Rivers (1936). A brief account only of the outstanding facts will be given here.

Watery extracts of vaccinia-infected tissue contain two specific components (Craigie, 1932). These may readily be separated by simple physical procedures, such as high-speed centrifugation, or filtration through a Seitz EK disc or collodion membrane of suitable porosity. The component deposited by centrifugation and held back by filtration, the "elementary bodies", carries all the infectivity of the virus. The supernatant or filtrate contains a substance which is non-infective, but which may be recognized by its specific reactions with anti-vaccinial sera. This substance is most probably a product of the elementary bodies. After being washed till they are apparently free of it they emit further amounts during subsequent storage (Craigie and Wishart, 1936). It may be referred to, for convenience and without commitment as to its actual nature, as a specific soluble substance, or "S.S.S."

Elementary bodies are specifically agglutinated (Ledingham, 1931) and the S.S.S. specifically precipitated (Tulloch, 1932) by sera obtained from animals convalescent from vaccinial infection or hyperimmunized with active virus. Both elementary bodies and S.S.S. fix complement with such sera (Craigie and Wishart, 1934*a*). A heat-stable and a heat-labile antigenic constituent are common to both elementary bodies and S.S.S., and have been shown to correspond to separate antibodies (Craigie and Wishart, 1934*b*, 1936*a*, 1936*b*), but they have not themselves been separated from one another except by inactivation of the labile constituent. Exposure to temperatures of 56° C. or to 1 p.c. formalin inactivates the labile constituents of both elementary bodies and S.S.S., as judged by failure to agglutinate or precipitate, as well as to absorb or induce the production of antibody. The stable constituents withstand boiling (but not autoclaving), and are unaffected by 1 p.c. formalin. With respect to these antigens, which are those chiefly concerned with agglutination and precipitation, elementary bodies and S.S.S. appear to be indistinguishable. There is, however, some evidence for the existence of other antigens, present in elementary bodies, but not in the S.S.S. (Craigie and Wishart, 1936*b*).

The effects of injecting elementary bodies and S.S.S., respectively, into animals differ significantly. When active elementary bodies are injected into normal susceptible animals symptoms of infection are followed by a rapid

production of agglutinin, precipitin, and complement-fixing and virus-neutralizing antibodies (Ledingham, 1932, 1933 ; Craigie and Wishart, 1936*c*), and the animal becomes immune to further infection for a variable period. When S.S.S. is injected into normal animals, agglutinin, precipitin, and complement-fixing antibodies are, in general, more slowly produced, and neutralizing antibody appears only in low titre, if at all ; moreover, the active immunity induced is of a low order or may be absent. Injection of elementary bodies inactivated by heat or formalin gives results very similar to injection of S.S.S. (Craigie, 1934 ; Parker and Rivers, 1936). Injection of either active or inactivated elementary bodies or of S.S.S. into animals previously vaccinated with active virus all give rise to an accelerated cutaneous reaction and to a rise in agglutinin, precipitin, and complement-fixing, titres (Ledingham, 1933 ; Craigie and Wishart, 1933, 1936*c*).

The fact that vaccines inactivated by heat or formalin are relatively ineffective in producing immunity to infection (Parker and Rivers, 1936) suggests that the labile antigen described by Craigie and Wishart (1934*b*) may be essential for this purpose. However, preparations of S.S.S. rich in the labile antigen also fail to produce more than low degrees of active immunity. It is possible that the labile antigen is only fully effective as an immunizing agent when it is attached to the elementary body. The immunizing properties of S.S.S. can be improved by adsorption on inert particles (Salaman, 1934).

The records of titrations of vaccinial antisera produced in response to different types of inoculum, such as are given by Parker and Rivers (1936), reveal a striking lack of parallelism between agglutinin, precipitin and virus-neutralizing titres. Agglutinin and precipitin titres appear to diverge slightly, but definitely, from proportionality to one another. Virus-neutralizing titres are not even approximately proportional to agglutinin or precipitin titres. The degree of immunity to infection bears no constant relation to agglutinin or precipitin titre, but appears to correspond roughly to virus-neutralizing titre.

SCOPE OF THE PRESENT WORK.

There appears to be little evidence to connect the agglutinogens and precipitinogens of vaccinia, or their antibodies, either with the phenomenon of virus-neutralization or with the mechanism of immunity. Does virus-neutralization depend upon an antigen-antibody union different from those concerned with agglutination or precipitation ?

It was thought that serum absorption might throw light on this question. Various anti-vaccinial sera were absorbed with vaccinial elementary bodies and with Seitz filtrates of vaccinial pulp extracts, respectively, and the absorbed sera tested for agglutinin, precipitin, and neutralizing antibody. No attempt was made to modify either elementary bodies or Seitz filtrates by physical or chemical means.

METHODS.

Preparation of Antigens.

Washed Elementary Body Suspensions.

The method used was substantially that of Craigie (1932), and need be only briefly described. The strain was originally derived from sheep-lymph (Lister Institute No. 1715, erroneously

described as calf-lymph in a previous paper [Salaman, 1934]), and had been through some 40 passages on rabbits' skin before the present work was begun. Third or 4th day rabbit dermal pulp was shaken, without grinding, with *M*/250 citric acid + disodium phosphate buffer, pH 7·1. After clarification by light centrifugation the elementary bodies were deposited on a Lundgren angle centrifuge. They were washed and redeposited, and resuspended in a quarter of the finally desired volume of buffer. This suspension was spun on an ordinary centrifuge at 3000 r.p.m. for ½ hour. The supernatant was pipetted off, and the deposit resuspended in a further quarter-volume and spun at 3000 r.p.m. for ½ hour; this was done four times in all. The final deposit was discarded. The total volume of the pooled supernatants was 10 c.c. for the yield of each rabbit. (This fractional resuspension, in respect of which the present method differs from Craigie's, effected a great saving of elementary bodies without sacrifice of purity). The whole process was carried out in one day, at room temperature; chilled buffer was used for each resuspension (Craigie ran the angle centrifuge in the cold room, but this has not been found necessary so far). The final suspensions were virulent intradermally in the rabbit in high dilution (10^{-6} to 10^{-8}), and showed remarkable homogeneity, and very fair freedom from cellular debris, under the microscope. They were stored at 0–4° C., and were used up too rapidly to require any preservative.

They will be referred to as "elementary body suspensions", or "E.Bs."

Specific Soluble Substance.

The supernatant from the first deposition of elementary bodies on the angle centrifuge was saved, NaCl was added to 0·85 p.c., and it was filtered through a Seitz EK disc previously treated with a 1 : 10 dilution of normal rabbit serum. The filtrate was tested for bacterial (aërobic) sterility, for precipitating power against a suitable serum, and for absence of active virus by multiple intradermal injections of 0·5 c.c. volumes into rabbits. Only filtrates satisfactory in these respects were used. (Very slight virulence, not more than a millionth of that of the corresponding elementary body suspension, was negligible for absorption experiments, but could be removed, when necessary, by high-speed centrifugation.) The filtrates were stored at 0–4° C. without preservative. They will be referred to as "Seitz filtrates", or "S.Fs."

Preparation of Antisera.

Hyperimmune Sera.

Rabbits, after being used for intradermal titrations of vaccinia, were given one or more weekly intravenous injections of virulent elementary body suspensions, and bled a week after the last injection. Serum from a number of such animals was pooled.

Convalescent Sera.

In certain cases samples of serum were collected from rabbits at various intervals after vaccination (see p. 249).

All sera were filtered through Berkefeld V candles, and stored at 0–4° C., without preservative, for at least a month before use.

Precipitation and Agglutination Tests.

Here, also, the method used was based on that of Craigie (1932). Either total volumes of 0·5 c.c. in ⅜-in. tubes, or of 0·2 c.c. in Durham tubes, according to the quantity of the reagents available, were used. Normal saline was the diluent throughout, with one exception: the antigen for the agglutination reaction was made by diluting a stock elementary body suspension 1 : 10 with saline containing 0·25 p.c. formalin and *M*/250 citric acid + disodium phosphate, pH 7·1. The tubes were closed with rubber stoppers and incubated at 50° C. for 18 hours. Normal serum and saline controls were included.

Virus Neutralization Tests.

The method of adding a constant dilution of serum to serial dilutions of an elementary body suspension was found more satisfactory in these experiments than the converse arrangement. The mixtures were allowed to stand for 1 hour at room temperature, then 0·2 c.c. of each was injected intradermally on a rabbit's flank. Sera to be compared were always tested on the same rabbit. Controls of normal serum and known anti-vaccinial serum were included.

Serum Absorption.

Early in the course of the present work it became clear that effective absorption of vaccinial antibodies depended on correct dosage of antigen. The precipitation of vaccinial Seitz filtrates by anti-vaccinial sera shows a well-marked Dean and Webb (1926), or "constant-serum", optimum, as has been recently pointed out (Craigie and Wishart, 1936*a*). The ratio of the concentration of the reagents in the optimal mixture is found to be constant. Tests of the supernatant fluid after precipitation show that this ratio corresponds closely to equivalence; that is to say, neither precipitin nor precipitating antigen are detectable in the supernatant after precipitation at the optimal ratio, while supernatants from mixtures originally containing more than the optimal proportion of Seitz filtrate contain residual precipitating antigen, and those from mixtures originally containing more than the optimal proportion of serum contain residual precipitin.

A comparable phenomenon was observed by the present writer in the agglutination of elementary bodies by anti-vaccinial sera. There is an optimal, *i. e.* most rapid, agglutination ratio, using the "constant serum" method, analogous to that described for bacteria (Duncan, 1932, 1934; Miles, 1933). This ratio also appears to correspond to equivalence, judged by tests of supernatant fluids for residual agglutinin.

The optimal agglutination ratio cannot be directly determined by a "constant-serum" test in ordinary tubes, owing to the great opacity of the higher antigen concentrations. A capillary-tube method was found satisfactory. Serial two-fold dilutions of an elementary body suspension were made in *M*/250 buffer pH 7·1. To each dilution was added an equal volume of a relatively high dilution (*e. g.* 1 : 10 or 1 : 20), in 0·25 p.c. formol-buffer-saline pH 7·1, of the antiserum to be absorbed. The tubes were shaken immediately, and portions from each drawn up into a long capillary, separated by air-spaces. The capillary was sealed and observed with a hand lens in indirect light. Agglutination at the optimal ratio began in 1 to 2 minutes at room temperature. Absorbing doses of elementary bodies were calculated on the basis of this ratio. When the dose had been fixed the required volume of elementary body suspension was centrifuged at 14,000 r.p.m. for ½ hour. (The supernatant fluid was tested for precipitation against the serum to be absorbed. Its titre was normally low; if it was as high as 1/10th of that of the corresponding Seitz filtrate the elementary bodies were washed again before use.) The deposited elementary bodies were well dispersed in the volume of serum, suitably diluted with saline, to be absorbed. The suspension was incubated at 37° C. for 1½ hours, together with a control dilution of the serum alone; and then centrifuged at 14,000 r.p.m. for 1 hour, to remove the elementary bodies. The absorbed serum was pipetted off. In certain cases (see p. 249) further doses of elementary bodies were applied and the process repeated.

The optimal precipitation ratio was determined by an ordinary Dean and Webb test in ⅜-in. tubes. Precipitation at the optimal ratio began in ¾ to 1½ hours at 50° C. Absorbing doses of Seitz filtrate were calculated on the basis of this ratio. When the dose had been fixed the required volume of Seitz filtrate was mixed with the volume of serum to be absorbed, together with enough saline to bring the serum to a suitable dilution. The conditions of absorption were the same as for elementary bodies, except that centrifugation was at 3000 r.p.m. for ½ hour, which was quite sufficient to remove the precipitates which formed. (It should be noted that S.S.S. can only be removed if it precipitates. Amounts in excess of the optimal precipitating dose cannot be removed [see p. 252]).

Absorbed sera were tested for residual agglutinin, precipitin, and neutralizing power.

RECORDS OF REPRESENTATIVE EXPERIMENTS.

Absorption of Hyperimmune Serum with Various Single Doses of Antigens.

A pooled hyperimmune anti-vaccinial serum (P_2) was prepared, as described above. 3 c.c. volumes of serum P_2, diluted 1 : 5 with saline, were absorbed with—

(*a*) one-third of an optimal agglutinating dose of E.Bs.;
(*b*) one optimal agglutinating dose of E.Bs.;
(*c*) three optimal agglutinating doses of E.Bs.;
(*d*) one-third of an optimal precipitating dose of S.F.;
(*e*) one optimal precipitating dose of S.F.;
(*f*) three optimal precipitating doses of S.F.

After incubation agglutination was most marked in (*b*), and precipitation most marked in (*e*); but some degree of agglutination or precipitation was visible in all.

The absorbed sera were tested for agglutinin and precipitin (Table I) and virus neutralizing power (Table II).

Absorption of Hyperimmune Serum with Successive Doses of Antigens.

A number of other absorbing doses of the antigens were tried. The absorptive capacity of a dose was found to be increased by applying it fractionally, as was to be expected; for instance, two optimal agglutinating doses of elementary bodies applied successively absorbed approximately as much neutralizing antibody as three applied at once. It was not found generally practicable to use a larger amount of elementary bodies than three optimal agglutinating doses. In order to make reasonably certain that absorption was complete, serum was absorbed with three optimal agglutinating doses of elementary bodies applied successively, the mixtures being incubated and centrifuged, as described on p. 248, after each fresh addition of antigen. The first dose agglutinated during incubation, the subsequent doses did not.

The same serum was also absorbed with three optimal precipitating doses of Seitz filtrate, applied successively. The first dose precipitated during incubation, the subsequent doses did not.

Three c.c. volumes of hyperimmune anti-vaccinial serum P_2, diluted 1 : 5 with saline, were absorbed with—

(*a*) one optimal agglutinating dose of E.Bs.;
(*b*) three successive optimal agglutinating doses of E.Bs.;
(*c*) one optimal precipitating dose of S.F.;
(*d*) three successive optimal precipitating doses of S.F.;

and tested for residual agglutinin and precipitin (Table III) and virus-neutralizing power (Table IV).

Absorption of Convalescent Sera.

Several other anti-vaccinial sera were absorbed, with uniformly similar results. An example will be given of the absorption of a serum of a different type from that used in the previous experiments. If the agglutinin, precipitin, and neutralizing, titres of the serum of a rabbit are tested at intervals after vaccination, the former two are usually found to fall off more rapidly than the last. Occasionally serum is obtained 3 to 4 months after vaccination which has fairly good virus-neutralizing power, but no detectable agglutinin or precipitin. Such a serum (26) was absorbed with doses of elementary bodies, and of Seitz filtrate, respectively, based on optimal ratios determined against an ordinary hyperimmune serum having rather higher neutralizing power. No agglutination or precipitation occurred during incubation.

Three c.c. volumes of anti-vaccinial serum 26 (obtained from a rabbit 3 months after vaccination), diluted 1 : 5 with saline, were absorbed with—

(*a*) one optimal agglutinating dose of E.Bs. (determined for serum P_2);
(*b*) two optimal agglutinating doses of E.Bs. (determined for serum P_2);
(*c*) one optimal precipitating dose of S.F. (determined for serum P_2);
(*d*) two optimal precipitating doses of S.F. (determined for serum P_2);

and tested for residual virus-neutralizing power (Table V).

TABLE I.—*Agglutinin and Precipitin Tests.*

Serum dilutions	+ E.B. suspension, 1 : 10. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	+ S.F., 1 : 4. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.
Unabsorbed serum P_2	+++	+++	++++	+++	+	−	++++	+++	++	−	−	−
Serum P_2 abs. ½ opt. E.Bs.	+++	++	−	−	−	−	++	+	±	−	−	−
,, ,, opt. E.Bs.	−	−	−	−	−	−	++	+	−	−	−	−
,, ,, 3 opt. E.Bs.	−	−	−	−	−	−	++	+	−	−	−	−
,, ,, ⅓ opt. S.F.	+++	++	+	−	−	−	+++	+	±	−	−	−
,, ,, opt. S.F.	++	+	−	−	−	−	−	−	−	−	−	−
,, ,, 3 opt. S.F.	−	−	−	−	−	−	−	−	−	−	−	−
Normal rabbit serum	−	−	−	−	−	−	−	−	−	−	−	−
Saline	−	..	..	..	..	..	−	..	..	..	..	..

Abbreviations: E.Bs. = Elementary bodies from vaccinial rabbit-skin pulp.
S.F. = Seitz filtrate of extract of vaccinial rabbit-skin pulp.
abs. = Absorbed with.
opt. = Optimal agglutinating or precipitating dose.

TABLE II.—*Virus-Neutralization Test.*

Dilutions of E.B. suspension.	+ Unabsorbed serum P_2, 1 : 5.	+ Serum P_2, 1 : 5. Abs. ½ opt. E.Bs.	+ Serum P_2, 1 : 5. Abs. opt. E.Bs.	+ Serum P_2, 1 : 5. Abs. 3 opt. E.Bs.	+ Serum P_2, 1 : 5. Abs. ½ opt. S.F.	+ Serum P_2, 1 : 5. Abs. opt. S.F.	+ Serum P_2, 1 : 5. Abs. 3 opt. S.F.	+ Normal rabbit serum. 1 : 5.
10^{-1}	6 mm.	12 mm.	15 mm.	17 mm.	7 mm.	9 mm.	7 mm.	16 mm.
10^{-2}	5 ,,	9 ,,	14 ,,	16 ,,	3 ,,	5 ,,	3 ,,	15 ,,
10^{-3}	—	5 ,,	13 ,,	16 ,,	—	—	—	14 ,,
10^{-4}	—	—	7 ,,	8 ,,	—	—	—	9 ,,
10^{-5}	—	—	—	5 ,,	—	—	—	5 ,,
10^{-6}	—	—	—	4 ,,	—	—	—	4 ,,

The figures in the 2nd to 9th columns represent diameters of the lesions in millimetres, measured on the 5th day after injection.
(Abbreviations as in Table I.)

TABLE III.—*Agglutinin and Precipitin Tests.*

Serum dilutions	+ E.B. suspension, 1 : 10. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.	+ S.F., 1 : 8. 1/5.	1/10.	1/20.	1/40.	1/80.	1/160.
Unabsorbed serum P_2	++++	++++	++++	+++	+	±	++++	+++	+	−	−	−
Serum P_2 abs. opt. E.Bs.	−	−	−	−	−	−	++	+	−	−	−	−
„ „ 3 successive opt. E.Bs.	−	−	−	−	−	−	+	±	−	−	−	−
„ „ opt. S.F.	++	+	−	−	−	−	−	−	−	−	−	−
„ „ 3 successive opt. S.F.	±	−	−	−	−	−	−	−	−	−	−	−
Normal rabbit serum	−	..	..	..	..	..	−	..	..	..	..	..
Saline	−	..	..	..	..	..	−	..	..	..	..	..

(Abbreviations as in Table I.)

TABLE IV.—*Virus-Neutralization Test.*

Dilutions of E.B. suspension.	+ Unabsorbed serum P_2, 1 : 5.	+ Serum P_2, 1 : 5. Abs. opt. E.Bs.	+ Serum P_2, 1 : 5. Abs. 3 successive opt. E.Bs.	+ Serum P_2, 1 : 5. Abs. opt. S.F.	+ Serum P_2, 1 : 5. Abs. 3 successive opt. S.F.	+ Normal rabbit serum, 1 : 5.
10^{-2}	15 mm.	19 mm.	20 mm.	5 mm.	14 mm.	18 mm.
10^{-3}	11 „	17 „	19 „	—	5 „	17 „
10^{-4}	9	13 „	16 „	—	—	16 „
10^{-5}	—	9 „	14 „	—	—	14 „
10^{-6}	—	2 „	14 „	—	—	12 „
10^{-7}	—	—	13 „	—	—	11 „
Virulence tests of absorbed sera, injected alone		—	—	—	—	

(Notation and abbreviations as in Tables I and II.)

Control Experiments.

Non-specific Inhibition of Agglutination and Precipitation by Absorbed Sera.

Craigie (1936*b*) has pointed out that anti-vaccinial sera absorbed with Seitz filtrates of dermal pulp extracts sometimes have the power of inhibiting precipitation when added to fresh mixtures of serum and Seitz filtrate. In view of this observation absorbed sera were tested for possible inhibitory effects on both agglutination and precipitation. Duplicate series of two-fold dilutions in saline were made of each absorbed serum to be tested. To each dilution of one series was added an equal volume of a freshly-made mixture of elementary bodies and untreated serum, in dilutions such as to give just definite agglutination. To the other series a similar mixture of Seitz filtrate and untreated serum was added. The tubes were incubated for 18 hours at 50° C. It was found that sera absorbed with elementary bodies had no inhibitory effect either on agglutination or on precipitation. Serum absorbed with an optimal precipitating dose of Seitz filtrate had a slight inhibitory effect on both agglutination and precipitation. Serum absorbed with larger doses of Seitz filtrate had a slight inhibitory effect on agglutination, and a marked, though not complete, inhibitory effect on precipitation.

It is apparent that the observed reduction of agglutinin and precipitin titres produced by absorption with excess of Seitz filtrate may have been, in part at any rate, an inhibition rather than a true absorption. (As excess of Seitz filtrate cannot be removed, it seems likely that these effects are examples of the familiar inhibition of agglutination or precipitation by excess of antigen, and should therefore not be called " non-specific "). In cases of absorption with optimal or smaller doses of Seitz filtrate, or with elementary bodies in any dose, the observed effects could not have been due to inhibition.

Effect of Skin Extracts on Infectivity of Vaccinia and on Virus-neutralizing Power of Sera.

Sera absorbed with Seitz filtrates of vaccinial skin-pulp contain considerable amounts of non-specific tissue extractives. In view of the slight apparent increase in neutralizing power of sera after absorption with Seitz filtrates (see Tables IV and V), the effects of these substances on the infectivity of elementary bodies and on their neutralization by anti-vaccinial sera were tested.

To each of similar series of ten-fold dilutions of an elementary body suspension equal volumes of one of the following were added :

(*a*) saline ;
(*b*) normal rabbit serum, 1 : 5 in saline ;
(*c*) ditto, + Seitz filtrate of extract of normal rabbit-skin scrapings ;
(*d*) ditto, + Seitz filtrate of extract of vaccinial rabbit-skin pulp ;
(*e*) anti-vaccinial serum P_2, 1 : 5 in saline ;
(*f*) ditto, + Seitz filtrate of extract of normal rabbit-skin scrapings.

TABLE V.—*Virus-Neutralization Test.*

Dilutions of E.B. suspension.	+ Unabsorbed serum P_2, 1 : 5.	+ Unabsorbed serum 26, 1 : 5.	+ Serum 26, 1 : 5. Abs. opt. E.Bs.	+ Serum 26, 1 : 5. Abs. 3 opt. E.Bs.	+ Serum 26, 1 : 5. Abs. opt. S.F.	+ Serum 26, 1 : 5. Abs. 3 opt. S.F.	+ Normal rabbit serum, 1 : 5.
10^{-1}	13 mm.	14 mm.	16 mm.	15 mm.	14 mm.	13 mm.	14 mm.
10^{-2}	12 „	12 „	14 „	14 „	9 „	12 „	14 „
10^{-3}	7 „	10 „	13 „	13 „	8 „	10 „	13 „
10^{-4}	—	5 „	9 „	12 „	—	—	13 „
10^{-5}	—	—	5 „	10 „	—	—	10 „
10^{-6}	—	—	4 „	6 „	—	—	5 „

(Notation and abbreviations as in Tables I and II.)

TABLE VI.—*Virus-Neutralization Test.*

Dilutions of E.B. suspension.	+ Saline.	+ Normal serum, 1 : 5.	+ Normal serum, 1 : 5, and S.F. normal skin.	+ Normal serum, 1 : 5, and S.F. vaccinial skin.	+ Anti-vaccinial serum P_2, 1 : 5.	+ Anti-vaccinial serum P_2, 1 : 5, and S.F. normal skin.
10^{-2}	16 mm.	15 mm.	15 mm.	16 mm.	5 mm.	10 mm.
10^{-3}	14 „	14 „	14 „	13 „	—	—
10^{-4}	9 „	14 „	14 „	12 „	—	—
10^{-5}	5 „	12 „	11 „	9 „	—	—
10^{-6}	—	5 „	—	—	—	—
10^{-7}	—	—	—	—	—	—

(Notation and abbreviations as in Tables I and II.)

TABLE VII.—*Virus-Neutralization Test.*

Dilutions of vaccinial E.B. suspension.	+ Unabsorbed serum P_2, 1 : 5.	+ Serum P_2, 1 : 5. Abs. vaccinial E.Bs.	+ Serum P_2, 1 : 5. Abs. normal skin suspension.	+ Serum P_2, 1 : 5. Abs. herpetic skin suspension.	+ Normal rabbit serum, 1 : 5.
10^{-1}	7 mm.	18 mm.	7 mm.	6 mm.	18 mm.
10^{-2}	6 „	17 „	7 „	4 „	17 „
10^{-3}	3 „	16 „	3 „	—	15 „
10^{-4}	—	7 „	—	—	11 „
10^{-5}	—	5 „	—	—	11 „
10^{-6}	—	4 „	—	—	7 „
10^{-7}	—	—	—	—	—

(Notation and abbreviations as in Tables I and II.)

(The proportions of serum and of Seitz filtrates were similar to those in sera absorbed with three optimal precipitating doses of Seitz filtrate.

The tubes stood for 1 hour at room temperature.

0·2 c.c. from each was injected intradermally, all on the same rabbit's back. The results are shown in Table VI.

Neither extracts of normal nor of vaccinial skin appeared to have any significant effect on the virulence of an elementary body suspension, nor did the former affect the neutralizing power of anti-vaccinial serum. Normal serum appeared to enhance virulence slightly, and though the difference in titre recorded in Table VI is within the limits of experimental variation, the constancy of this effect suggested that it was significant.

Effect of Absorbing Anti-vaccinial Sera with Heterologous Antigens.

Although the elementary body suspensions used in these experiments showed microscopically a high degree of homogeneity, there was always present a small amount of cellular debris. It was necessary to exclude the possibility that non-specific absorption by tissue debris was responsible for the observed removal of virus-neutralizing power by elementary body suspensions.

Two control antigens were used :

(*a*) *Suspension obtained from normal skin.*—Scrapings of normal rabbit-skin were treated in the same way as vaccinial pulp, and suspensions were obtained which, though of considerably less opacity than vaccinial elementary body suspensions, contained an appreciable amount of suspended matter.

(*b*) *Suspension obtained from herpetic skin.*—A strain of herpes virus, which had been passed in the pads of guinea-pigs, was obtained through the kindness of Prof. S. P. Bedson, of the London Hospital, E. 1. This was adapted to rabbits' skin, a satisfactory take being obtained at the second passage. A suspension made from this herpetic rabbit-skin pulp was of similar opacity to vaccinial elementary body suspensions. Its virulence was found to be neutralized by an anti-herpetic serum, but unaffected by an anti-vaccinial serum.

Samples of hyperimmune anti-vaccinial serum were absorbed with each of these suspensions, by the method described on p. 248. In each case an absorbing dose was used considerably in excess, calculated as a proportion of the total yield of a rabbit, of the dose of vaccinial elementary bodies required to absorb a similar amount of the serum.

In the heterologous suspension + serum mixtures no detectable aggregation occurred during incubation.

Three c.c. volumes of hyperimmune anti-vaccinial serum P_2, diluted 1 : 5 with saline, were absorbed with—

(*a*) two successive optimal agglutinating doses of vaccinial E.Bs. (2/3rds of the yield of one rabbit to 1 c.c. of serum) ;

(*b*) two successive doses of suspension from normal skin (the yield of 2 rabbits to 1 c.c. of serum) ;

(*c*) two successive doses of suspension from herpetic skin (the yield of 1 rabbit to 1 c.c. of serum) ;

and were tested for residual virus-neutralizing power (Table VII).

DISCUSSION OF RESULTS.

The results of which representative examples have been given may be briefly stated as follows:

Absorption of Hyperimmune Anti-vaccinial Serum.

Tables II and IV: Neutralizing power is progressively removed by absorption with increasing doses of washed elementary bodies obtained from vaccinial rabbits' skin pulp. It is definitely reduced by one optimal agglutinating dose. Two successive or three simultaneous optimal doses are required to remove it completely. It is unaffected by absorption with three successive optimal precipitating doses of Seitz filtrate of an extract of the same pulp.

Tables I and III: Agglutinating power is progressively removed by absorption with increasing doses of elementary bodies. One optimal agglutinating dose removes it completely. It is partially removed by absorption with Seitz filtrate; one optimal precipitating dose reduces its titre to about 1/8th of the original. Larger doses may appear to remove it completely, but here the possibility of inhibition of agglutination by excess of Seitz filtrate must be borne in mind (see p. 252).

Precipitating power is partially removed by absorption with elementary bodies; one optimal agglutinating dose reduces its titre to about half the original, but larger doses do not reduce it much further. It is progressively removed by absorption with increasing doses of Seitz filtrate. One optimal precipitating dose removes it completely.

Absorption of 3-months Convalescent Anti-vaccinial Serum, having Virus-neutralizing but no Agglutinating or Precipitating Power.

Table V: Neutralizing power is removed completely by absorption with a suitable dose of elementary bodies, but is unaffected by absorption with a comparable dose of Seitz filtrate.

Control experiments *in vitro* excluded the possibility of an inhibitory effect simulating agglutinin and precipitin absorption, except in the case of absorption with excess of Seitz filtrate.

Tables VI and VII: Control experiments *in vivo* excluded the possibilities of non-specific absorption of neutralizing power, or of non-specific interference with its detection.

A point which is not evident from the tabulated results is worth noting. The dose of elementary bodies required to remove the neutralizing power of a certain quantity of serum by absorption is a high multiple of the dose the virulence of which the same quantity of serum can neutralize. The ratio between these doses varies considerably; for fresh suspensions it is never less than 1500 to 1, and may be as high as 100,000 to 1. The dose required to effect just detectable absorption is not less than a third of the full absorbing dose.

The salient fact which emerges is that washed elementary bodies are able to remove neutralizing power from a serum, but Seitz filtrate is not. Taken together with evidence of the independence of neutralizing and agglutinin titres, already referred to, this observation supports the view that neutralizing

power depends upon a separate antibody. If this is true, we may expect to find a corresponding antigen, distinct from the agglutinogens and precipitinogens. Until such an antigen has been distinguished and its properties defined, it would be unwise to draw definite conclusions.

Less striking, but definite and regularly reproducible, are the differences in the absorptive powers of these antigens for agglutinin and precipitin respectively. A dose of elementary bodies which absorbs all agglutinin leaves residual precipitin, and a dose of Seitz filtrate which absorbs all precipitin leaves residual agglutinin. Discussion of these observations will be postponed until they have been correlated with those of Craigie and Wishart (1936*b*) on the stable and labile agglutinogen and precipitinogen.

There has been a good deal of controversy as to whether viruses are able to unite with their antibodies *in vitro*. The bearing of the results above recorded on this question will be briefly considered.

The ability of vaccinial elementary bodies and S.S.S. to absorb agglutinin and precipitin is well established, and this must be regarded as evidence of union *in vitro*. Conflict of opinion has mainly concerned union *in vitro* between virus and neutralizing antibody. Much of the earlier work supplied evidence of such union, *e. g.* Bedson (1929) showed that collodion particles which had been treated with anti-herpes serum adsorbed herpes virus more strongly than collodion particles treated with normal serum, and Wilson Smith (1930) was able to remove the neutralizing power of a serum by absorption with vaccinial rabbit testicle. On the other hand, the reactivation by dilution, filtration, or selective adsorption, of apparently neutral serum-virus mixtures, unless previously incubated for long periods (Bedson, 1928 ; Andrewes, 1928 ; Todd, 1928), and the extraordinary differences in virulence of serum-virus mixtures when injected into different tissues (Andrewes, 1928 ; Todd, 1928 ; Fairbrother, 1932), left an impression in the minds of many workers that union *in vitro*, if it occurred, must be easily reversible, and that the observed facts of virus neutralization could not be fully accounted for by such a union.

Sabin (1935*a*, *b*, *c*, *d*), in a series of researches on immunity to vaccinia, herpes, " B virus ", and pseudorabies, obtained results which led him to conclude that no union of virus and neutralizing antibody occurs *in vitro*, and that immunity to viruses in general is in some way bound up with properties of living tissue not at present understood. His basic experiments were as follows :

1. Virulent Berkefeld filtrates of vaccinial rabbit testicle extracts were mixed with anti-vaccinial sera in such proportions that the mixtures were avirulent on intradermal injection ; from the data given it appears that a slight excess of serum was present. The mixtures were incubated for varying periods, and then the virus was deposited by high-speed centrifugation. The supernatants were removed. The deposits were resuspended in saline to the original volume, and were found to be unreduced in virulence, as compared with deposits from normal serum-virus mixtures. Prolonged incubation of the mixtures did not abolish the virulence of the deposited virus. Similar results were obtained with B virus and pseudorabies.

2. A similar vaccinial Berkefeld filtrate and anti-vaccinial serum were mixed in such proportions that the mixture was virulent on intradermal injection ; the virulence, as recorded, was not great, and indicated a small excess of virus only. The mixture was incubated for 4 hours at 37° C. and 24 hours at room temperature, and was then filtered through a Seitz disc. The filtrate was found to have unreduced virus-neutralizing power, as compared with a corresponding dilution of untreated serum.

Sabin's first experiment has been repeated in its essentials by the present writer, using dermal vaccinia, and his results have been confirmed. Virus spun out from neutral, or just over-neutralized, serum-virus mixtures is still virulent. But when as the proportions of the reagents are altered different results are obtained. A progressive increase in the proportion of serum brings about a progressive decrease in the virulence of the deposited virus. Further investigation of this point is in progress.

Sabin's second experiment appears at first sight to conflict with those recorded in the present paper. The difference between the strains of vaccinia used is unlikely to be the cause of the discrepancy. Sabin's Berkefeld filtrates contained both elementary bodies and S.S.S. Of these, as has been shown, only the former are able to remove neutralizing power. Was the dose of elementary bodies present in Sabin's "excess virus" mixture sufficient to effect detectable absorption? From Sabin's data one may safely reckon that this dose was not greater than five times that which the serum could neutralize. The observation recorded on p. 225, regarding the size of absorbing doses, makes it clear that Sabin was using a dose at least a hundred times too small for detectable absorption, and three hundred times too small for complete absorption, of neutralizing antibody.

Sabin also reports an experiment (1935*b*) in support of his contention that antiviral immunity is primarily a function of tissue, in which an anti-vaccinial serum was absorbed with minced normal rabbit testis. A definite though incomplete removal of virus-neutralizing power was obtained. On examination of his data it appears that 2 g. of testis were added to 2 c.c. of a 1 : 10 dilution of serum. An *ad hoc* experiment was carried out to determine the weight of moist packed elementary bodies required to remove the neutralizing power completely from this amount of a serum similar to Sabin's; it was approximately 20 mg. That 100 times this weight of normal tissue effected only partial removal is, in fact, good additional evidence of the specificity of absorption by elementary bodies.

SUMMARY.

1. Experiments are described in which anti-vaccinial serum was absorbed with vaccinial elementary bodies, and with vaccinial "specific soluble substance" respectively.

(*a*) Absorption with elementary bodies removes the virus-neutralizing power of a serum, but absorption with S.S.S. does not remove it.

(*b*) Absorption with elementary bodies removes all agglutinin, but not all precipitin, from a serum; absorption with S.S.S. removes all precipitin, but not all agglutinin.

(*c*) Serum obtained 3 to 4 months after vaccination sometimes has virus-neutralizing power without detectable agglutinin or precipitin. Its virus-neutralizing power can be removed by absorption with elementary bodies, but not by absorption with S.S.S.

2. Control experiments are described which confirmed the specificity of these effects.

3. The results are discussed, and the importance of correct absorbing doses

of antigen stressed. The hypothesis is tentatively advanced that virus-neutralization by anti-vaccinial sera depends on an antibody distinct from those responsible for agglutination and precipitation.

I wish to express my thanks to Sir John Ledingham and to Dr. A. Felix for much valuable advice.

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FOWL PEST: THE SUSCEPTIBILITY OF MONKEYS, HEDGEHOGS AND OTHER ANIMALS.

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IN previous communications (Mackenzie and Findlay, 1937; Findlay and Mackenzie, 1937) it was shown that two variant strains of fowl-pest virus could be produced by continued passage in the brains of canaries and mice from a naturally-occurring strain, isolated in this country. These variants, referred to as the canary and mouse strains, were distinguished from each other and from the parent strain, and also from a strain originally isolated by Prof. Doerr in Switzerland, by their behaviour when inoculated by various routes into mice and ferrets.

In the present communication an account is given of the transmission of fowl pest to rhesus monkeys, hedgehogs, ducks and pigeons.

FOWL PEST IN RHESUS MONKEYS.

Previous attempts to infect monkeys have been few. Krumwiede, Gerber and Provost (1925) injected monkeys subcutaneously, and fed them by mouth with material infected with what was believed to be the virus of fowl pest; the monkeys remained in good health. In the present experiments 16 rhesus monkeys were inoculated with the virus of fowl pest by the intraperitoneal, intranasal or intracerebral routes (Table I).

TABLE I.—*Inoculation of Rhesus Monkeys with the Virus of Fowl Pest.*

No.	Strain of virus.	Route of inoculation.	Result.
228	Doyle	i.c.	†11th day.
270	,,	i.p.	No fever; no symptoms.
264	,,	i.n.	,, ,,
224	Mouse	i.c.	†7th day.
252	,,	,,	Fever 2–7 days; recovered.
254	,,	,,	Killed 7th day; paralysis.
241	,,	i.p.	No fever; no symptoms.
263	,,	,,	,, ,,
242	,,	i.n.	,, ,,
225	,,	,,	,, ,,
269	Canary	i.c.	†7th day.
283	,,	i.p.	No fever; no symptoms.
284	,,	i.n.	,, ,,
281	Doerr	i.c.	Fever 3–9 days; recovered.
282	,,	i.p.	No fever; no symptoms.
286	,,	i.n.	,, ,,

i.c. = Intracerebral inoculation.
i.p. = Intraperitoneal inoculation.
i.n. = Intranasal instillation.
† = Death.

Except in Monkey 254 the inoculum consisted of the livers of hens infected with Doyle and Doerr strains of fowl pest, and canary and mouse brains infected with the two variant strains. The monkeys which were inoculated intraperitoneally and intranasally showed a complete absence of reaction, there being no rise of temperature during the 3 weeks following the inoculation. The 6 monkeys infected intracerebrally developed a febrile reaction which in the majority was followed by nervous symptoms, 4 of the animals becoming moribund between the 7th and 11th days. One, Monkey 281, which had been inoculated with the relatively avirulent Doerr strain showed only a slight febrile reaction.

The following are the protocols of the monkeys inoculated intracerebrally:

Monkey 228, inoculated intracerebrally with 1 c.c. of a 10 p.c. suspension of the liver of a hen moribund after infection with the Doyle strain of fowl-pest virus. The temperature rose to 104° F. on the 3rd day after inoculation, and remained elevated till the 8th day. Weakness developed in the hind legs on the 5th day after inoculation and progressed till the animal was killed on the 11th day, by which time an extreme paresis had involved all the limbs and the muscles of the neck. At post-mortem aërobic and anaërobic cultures of the heart and brain showed no bacterial growth. A small bronchopneumonic patch was present in the right lung.

Monkey 224, inoculated intracerebrally with 1 c.c. of a 10 p.c. suspension of mouse brain infected with the mouse strain of fowl-pest virus. The temperature rose to 104·6° F. 3 days after inoculation, and remained between 105°–106° F. till the 7th day, when the monkey was killed. Paresis of the legs appeared 5 days after inoculation and progressed very rapidly to the arms. At post-mortem aërobic and anaërobic cultures of blood and brain were again bacteriologically sterile. The liver was slightly pale, otherwise there were no naked-eye changes.

Monkey 252, inoculated as for Monkey 224. The temperature rose to 104° F. 2 days after inoculation and remained between 104° and 106° F. till the 8th day after injection, when it fell to 102·2° F. From the 3rd to the 7th day paresis of the legs was present, the monkey sitting huddled in its cage. The weakness did not spread to the upper limbs and the animal gradually recovered. Some 6 weeks after inoculation a left internal strabismus was noted, and somewhat later the monkey developed a tic-like movement of the neck, which was jerked from right to left.

Monkey 269, inoculated intracerebrally with 1 c.c. of a 10 p.c. suspension of canary brain infected with the canary strain of fowl-pest virus. On the 4th day after inoculation paresis began in the hind legs; the symptoms progressed, and in addition there was very pronounced tremor of the head and arms. The temperature rose to 104° F. on the 5th day after inoculation. On the 6th day the respirations were rapid and the monkey looked extremely ill. Death occurred on the 7th day after injection. At the post-mortem the left lung was dark red and solid; the lower lobe of the right lung was also consolidated. The kidneys were very pale yellow. The liver was brownish red. There was no evidence of tuberculosis. Smears from the lung failed to show any gross bacterial infection.

Monkey 281, inoculated intracerebrally with 1 c.c. of a 10 p.c. suspension of liver of hen infected with the Doerr strain of fowl-pest virus. The temperature rose to 104·6° F. on the 4th day after inoculation, and did not sink below 104° F. till the 9th day. The monkey showed no paresis and at no time looked ill.

Monkey 254, inoculated intracerebrally with 1 c.c. of a 20 p.c. suspension of the brain of Monkey 224, which in turn had been infected with the mouse strain of virus. The temperature rose to 104·8° F. on the 3rd day after inoculation, and remained between 104° and 105° F. for the next 48 hours. On the 5th day a watery catarrhal discharge was noted from both nostrils, which later became viscid and yellow. On the 6th day weakness appeared in the legs, and 24 hours later was so pronounced that the animal could not rise from the floor. The monkey was killed on the 7th day after inoculation, when the temperature had fallen to 100·8° F. No characteristic naked-eye changes were found *post mortem*; aërobic and anaërobic blood-cultures were bacteriologically sterile.

Distribution of Virus in the Tissues of Monkeys.

A feature of the distribution of the fowl-pest virus in monkeys was the disappearance of the agent from the blood-serum whatever the route of inoculation. Thus, Monkey 242, inoculated intranasally with the mouse strain, showed virus present in the serum in small amounts 24 and 48 hours after nasal instillation (2 of 6 and 1 of 6 mice killed by intracerebral injection), but absent 4 days after inoculation. In Monkey 252, inoculated intracerebrally with the mouse strain, the blood-serum contained virus 2 days, but not 6 days later. Numerous observers have previously suggested that the virus in fowls is attached to the red cells; it seems possible that in monkeys it may also be attached to the formed elements. The distribution of the virus at death in 3 monkeys is shown in Table II. Its presence was determined by the

intracerebral injection of batches of 6 mice, except in Monkey 228, where it was detected by intramuscular injection in hens.

TABLE II.—*Distribution of Fowl-pest Virus in the Tissues of Monkeys.*

No.	Strain.	Days till death.	Distribution of virus. Blood-serum.	Blood-cells.	Brain.	Liver.	Spleen.	Adrenal.
			Mice.*					
224	Mouse	7	0	1	0	2	3	1
254	,,	7	0	2	3	3	6	0
			Hens.†					
228	Doyle	11	0	—	1	1	1	0

* Figures indicate the number of mice out of 6 dying of fowl pest.
† One hen inoculated intramuscularly with each tissue.

Virus was thus absent from the blood-serum at death, but was always present in association with the blood-cells and the liver and spleen. In Monkey 224 the virus could not be detected in the brain, although encephalitic lesions were present—an example apparently of autosterilization. In all those monkeys which failed to succumb to infection immune bodies against fowl-pest virus could be demonstrated, as detailed in a previous communication (Mackenzie and Findlay, 1937). Rhesus monkeys are thus susceptible to fowl-pest provided the virus comes in contact in sufficient amounts with the tissues of the central nervous system.

FOWL PEST IN HEDGEHOGS.

Hedgehogs are now known to be susceptible to a number of viruses as diverse as foot-and-mouth disease, rabies, pseudo-rabies, yellow fever and influenza. The following observations show that they can also be infected with fowl pest :

Intracerebral Inoculation.

Four hedgehogs were inoculated intracerebrally with 0·05 c.c. of a 20 p.c. suspension of mouse brain infected with the mouse strain of fowl-pest virus. All the hedgehogs became irritable, scratching themselves frequently, and in 2 there were symptoms of slight nasal discharge and snuffles, the exudate being clear and watery. These two hedgehogs died 12 and 14 days after inoculation, the only naked-eye pathological changes being broncho-pneumonic patches in the lungs. The distribution of virus in the tissues of the hedgehogs was determined by inoculation of a 10 p.c. suspension of the organs into the brains of mice. Virus in both animals was absent from the blood-serum, but present in the brain, liver and spleen.

Intraperitoneal Inoculation.

Three hedgehogs were inoculated intraperitoneally with 0·5 c.c. of a 20 p.c. suspension of mouse brain infected with the same strain of fowl-pest virus. Two of the animals showed no evidence of illness, but the third developed a slight nasal discharge 4 days after inoculation and was killed 3 days later, although it would almost certainly have survived, as did the other two

hedgehogs injected intraperitoneally. The only pathological change detected at post-mortem in this hedgehog was a small broncho-pneumonic patch near the apex of the right lung. On testing as before, fowl-pest virus was found in the liver and spleen, but not in the blood-serum or brain. Hedgehogs may thus be infected with the virus of fowl pest and may die if the injection of virus is made into the brain.

FOWL PEST IN PIGEONS.

The susceptibility of pigeons to fowl pest is usually regarded as very slight, and is one of the points by which the virus of fowl pest may be differentiated from that of Newcastle disease (*cf.* Doyle, 1935, and Manninger, 1936). Balozet (1934), however, has found, in confirmation of the observations of Kraus and Doerr (1908), that, though no symptoms occurred after a primary intracerebral inoculation of fowl pest into pigeons, the virus was recoverable 5 to 8 days later. After a second intracerebral passage the virulence increased and nervous symptoms began to appear. By the tenth intracerebral passage death regularly occurred in from 6 to 8 days, and after further passage the incubation period became even shorter and death took place in 2 or 3 days. Somewhat similar results had been obtained by Jouan and Staub (1920), who increased the virulence of fowl-pest virus by the inoculation of very young pigeons.

In a small number of experiments we, too, have been able to show that the pigeon may be susceptible to certain strains of fowl-pest virus, more especially on intracerebral inoculation. It will be seen from Table III that, though nervous symptoms, such as lack of balance and chorea-like movements of the head and neck developed, death did not necessarily follow in every instance. From the brains of the pigeons that died of the infection the virus could be successfully retransferred to the brains of mice. It is perhaps worthy of note that nervous symptoms were most common after inoculation with the canary strain which had been carried continuously in avian brains, while the mouse strain, which had been passaged in mammalian brains, only produced nervous symptoms in one of the 8 pigeons inoculated.

FOWL PEST IN DUCKS.

Ducks are usually regarded as being susceptible to the virus of fowl pest, but no very satisfactory account is given of duck epizootics (*cf.* Freese, 1908; Rachad, 1934). We have, however, noted an account of a curious outbreak recorded by Cominotti (1916) in which some 50 p.c. of the ornamental ducks *Anitra germana* kept in the Public Gardens in Milan were attacked. The virus at death could only be found in the central nervous system and was readily transmissible to fowls, but for some rather obscure reason domestic ducks were insusceptible.

The result of inoculating ducks (Indian Runner strain) with the Doyle, mouse and canary strains of fowl-pest virus is shown in Table IV. The inoculum as before consisted of an emulsion of hen liver in the case of the Doyle strain, and an emulsion of brain in the case of the mouse and canary strains. While no symptoms were produced on intramuscular injection, intracerebral inoculation usually caused very pronounced nervous symptoms ending in death. Two days after inoculation weakness in the legs was noted

and the gait became stumbling. Next there was weakness of the wings, which remained outstretched; later there was head-retraction and convulsive attacks, during which the neck was twisted into unnatural attitudes.

At death the blood, liver and brain were tested as before for the presence of fowl-pest virus; none could be found in the blood and liver, but virus was invariably present in the brain.

TABLE III.—*Inoculation of Pigeons with the Virus of Fowl Pest.*

No.	Strain.	Route of inoculation.	Result.
1	Doyle	i.c.	†4 days.
2	,,	,,	Nervous symptoms; survived.
3	,,	i.p.	,, ,, ,,
4	,,	,,	No symptoms; survived.
5	Mouse	i.c.	†5 days.
6	,,	,,	No symptoms; survived.
7	,,	,,	,, ,,
8	,,	,,	,, ,,
9	,,	i.p.	,, ,,
10	,,	,,	,, ,,
11	,,	,,	,, ,,
12	,,	,,	,, ,,
13	Canary	i.c.	†5 days.
14	,,	,,	Nervous symptoms; survived.
15	,,	,,	,, ,, ,,
16	,,	i.p.	,, ,, ,,
17	,,	,,	,, ,, ,,
18	,,	,,	No symptoms; survived.

i.p. = Intraperitoneal inoculation.
i.c. = Intracerebral inoculation.
† = Death.

TABLE IV.—*Inoculation of Ducks with Fowl Pest.*

No.	Strain.	Route of inoculation.	Result.
1	Doyle	i.c.	†3rd day; encephalitis.
2	,,	,,	Survived; no symptoms.
3	,,	i.m.	,, ,,
4	,,	,,	,, ,,
5	Mouse	i.c.	†3rd day; encephalitis.
6	,,	,,	†4th day; ,,
7	,,	i.m.	Survived; no symptoms.
8	,,	,,	,, ,,
9	Canary	i.c.	†3rd day; encephalitis.
10	,,	,,	,, ,,
11	,,	i.m.	Survived; no symptoms.
12	,,	,,	,, ,,

i.c. = Intracerebral inoculation.
i.m. = Intramuscular inoculation.
† = Death.

DISCUSSION.

The above observations show that the virus of fowl pest is pathogenic, not only for fowls, but for such animals as the rhesus monkey, hedgehog, pigeon and duck. In addition Doerr and Seidenberg (1931–32) have found that the fowl-pest virus may persist and probably multiply for a few days in the brains of guinea-pigs and rabbits, producing an inapparent infection. Our attempts to infect the rabbit and guinea-pig have not been numerous, but one very young rabbit succumbed to intracerebral inoculation, dying with symptoms of encephalitis 8 days after injection. Two young guinea-pigs also died with encephalitic symptoms after intracerebral inoculation, but attempts to passage the virus in these animals failed. In monkeys, hedgehogs and ducks death occurred only when the virus gained entrance to the central nervous system. In monkeys intranasal instillation produced immunity, but no febrile or nervous reaction, suggesting that the causal agent must reach the brain in considerable amounts if it is to produce anything more than a subclinical infection. It is perhaps for this reason that no accounts have yet been published of human infections with the virus of fowl pest.

SUMMARY.

1. Rhesus monkeys are susceptible by intracerebral inoculation to certain strains of the virus of fowl pest. They react with fever, and generally symptoms of an ascending myelitis are present. Virus is present at death in the brain, liver and spleen, and in association with the formed elements of the blood, but not in the blood-serum.

2. Rhesus monkeys inoculated intraperitoneally or intranasally do not show any reaction, but subsequently develop immune bodies.

3. Hedgehogs are susceptible to the virus of fowl pest when injected intracerebrally.

4. Ducks are also susceptible on intracerebral but not on intramuscular injection. Pigeons may develop nervous symptoms after intraperitoneal and intracerebral inoculation, but only die when virus is inoculated by the latter route.

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